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Particles Down-Regulates Airway Allergic Responses

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This is an annual report of the 2nd grant year. PI and 2 Research Associates moved to the current Institute September 2003 from East Carolina University, Greenville, NC and requested transfer of grantee institute. The request was approved on December 1, 2004. The project was re-started in December 2004 with a planned end date of March 23, 2007. Since there was no funding for 14 months during the transfer process, our progress has been limited. We requested a no-cost extension until May 23, 2008, addressed to Pat McAllister, USA MED RESEARCH ACQ ACTIVITY Office, on January 6 2005. This is pending. We established that our chitin particle preparations induce cytokines that down-regulate allergic immune responses. Although other immunomodulators including bacterial endotoxin (LPS), DNA and cell walls (HK-BCG) also induce similar profiles of cytokine production, they additionally induce splenic PGE₂-macrophage formation and the production of IL-10, a pro-inflammatory cytokine. Interestingly, all these agonists including chitin particles induce mitogen- activating protein kinases (MAPK) consisting of p38, JNK and Erk1/2. We found that, among agonists, cytokine production is mediated differentially by MAPK families

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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusions.....	8
References.....	8
Appendices.....	10

INTRODUCTION:

This annual report includes a brief summary of the 2nd year research and related activities supported by DAMD17-03-1-0004.

This project funded by DOD DAMD17-03-1-0004 was initiated at East Carolina University on February 24, 2003. PI and two research associates moved to the current institute, Florida Atlantic University (FAU), on September 30, 2003, and requested grant transfer. The transfer of grantee institute was approved on December 1, 2004. Since then, the project has re-started without changing the project ending day, March 23, 2007. Therefore, our progress has been limited. Furthermore, we have requested no-cost extension until May 23, 2008, addressed to Pat McAllister, USA MED RESEARCH ACQ ACTIVITY Office, on January 6, 2005. No-cost extension approval is pending. During the 14 month grant transfer period some funding has been available from a start up program provided by FAU.

Two additional postdoctoral research associates, Shoutaro Tsuji, Ph.D. and Makiko Y. Tsuji, DVM, Ph.D. will join the laboratory and conduct this project starting in April 2005. Also, a Ph.D. graduate student is being sought for the project.

BODY:

Task 1: To determine if oral administration of 1 – 4 μ m particles of chitin will down-regulate airway hyperreactivity (AHR) and GATA-3 levels as a measure of Th2 responses, and enhance T-bet levels as a measure of Th1 responses in the lungs of mice that are sensitized with ragweed allergens.

- a. Establish the effects of dose response of chitin particles (Months 1 – 4).*
- b. Establish therapeutic/prophylactic effects of chitin (Months 4 – 9).*
- c. Determine duration of chitin treatments (Months 8 – 12).*

We established that 1 – 10 μ m chitin preparations possess reliable Th1 adjuvant activities. Previous studies (1) indicating the down-regulation of airway allergic responses by chitin treatment were presented at the CDMRP's Military Health Research Forum (MHRF), 4/25-4/28/04, 2004, San Juan, Puerto Rico. Two additional abstracts have been prepared. We will continue to define the mechanisms, the dose-responses, and the therapeutic/prophylactic effects of chitin treatment in the following years.

Task 2: To determine if the effects of 1 – 4 μ m particles of chitin on endogenous IL-12- or IFN γ - mediated down-regulation of airway allergic responses will be greater than those of HK-BCG, ODN-CpG or an equal number of particles of 1 – 10 μ m chitin.

- a. Establish comparative studies on the effects of 1 – 4 μ m chitin, 1 – 10 μ m chitin, HK-BCG and ODN-CpG (Months 12 – 24).*
- b. Study if endogenous IL-12 or IFN γ is required for the chitin-induced down-regulation of GATA-3-mediated allergic responses (Months 24 – 40).*

We are seeking to establish that splenic PGE₂-M ϕ down-regulate the activity of HK-BCG as a Th1 adjuvant. Two abstracts and three manuscripts have been prepared. One will be published in the Journal of Leukocyte Biology. A summary of the findings is below.

Different populations of mononuclear phagocytes (M ϕ) show considerable diversity of cellular function including PGE₂ biosynthesis. Certain bacterial components enhance PGE₂ biosynthesis differentially in selected populations of M ϕ . IL-10 is proposed to inhibit PGE₂ biosynthesis by down-regulating prostaglandin G/H synthase-2 (PGHS-2) expression. To assess whether IL-10 regulates PGE₂ biosynthesis and PGHS-2 expression, splenic and bone marrow M ϕ were isolated from IL-10- deficient (IL-10^{-/-}), C57Bl/6 (wild type [WT] control) and Balb/c (comparison control) mice and treated with LPS and/or IFN γ as a model of bacterial

inflammation. LPS-induced PGHS-2 expression was similar for splenic MØ isolated from the three strains of mice. However, PGE₂ released by LPS-treated splenic MØ was significantly higher in IL-10^{-/-} and Balb/c than in WT cells. In the presence of LPS and IFN γ , PGHS-2 expression and PGE₂ release by both IL-10^{-/-} and Balb/c splenic MØ were enhanced compared to stimulation with LPS or IFN γ alone. However, there was no significant increase in PGE₂ release from WT splenic MØ treated with LPS plus IFN γ despite increased PGHS-2 expression. In sharp contrast, PGHS-2 expression and PGE₂ release by bone marrow MØ were greatly enhanced in IL-10^{-/-} cells compared to control cells. Our results indicate that IL-10 regulation of MØ PGE₂ biosynthesis and PGHS-2 expression are compartment-dependent, and that PGE₂ production is not directly linked to PGHS-2 levels. Furthermore, our findings emphasize strain-specific differences between C57Bl/6 and Balb/c mice with Balb/c appearing more similar to the IL-10^{-/-} than to the C57Bl/6 with respect to PGE₂ production.

Task 3: To determine if MØ will phagocytose more particles and produce more IL-12 in response to 1 – 4 μ m chitin compared to 1 – 10 μ m chitin.

a. Determine if MØ treated with 1 – 4 μ m chitin particles phagocytose more particles than when treated with an equivalent number of 1 – 10 μ m chitin particles (Months 41 – 44).

b. Determine if 1 – 4 μ m chitin particles induce more production of IL-12 than an equivalent number of 1 – 10 μ m chitin particles (Months 45 – 48).

Cellular events of phagocytosis that are associated with chitin-induced production of anti- and pro-inflammatory cytokines were characterized. Abstract “Mechanism of phagocytosed particle-induced IL-12 production in macrophage,” was presented at the 2004 Experimental Biology, 4/17-4/21/04, Washington DC. Figure 1 summarizes findings that MØ phagocytose chitin particles with activation of MAPK (mitogen-activated protein kinases), consisting of p38, Erk1/2 and JNK. Our studies employing specific MAPK inhibitors indicated that MAPK activation appears to regulate production of the three cytokines, TNF α , IL-1 β and IL-10. Figure 2 summarizes how each MAPK family mediates selected cytokine production. A summary of these findings is below.

Figure 1. 1 – 10 μ m chitin particles induced phosphorylation of p38, Erk1/2 and JNK (all MAPK families). Murine macrophage-like RAW264.7 cells at 1×10^6 cells/ml were stimulated with 1 – 10 μ m chitin particles (1,000 μ g/ml), soluble chitin oligosaccharide (1,000 μ g/ml), > 50 μ m chitin particles (1,000 μ g/ml), 1 – 10 μ m chitosan particles (1,000 μ g/ml), 1.1 μ m latex beads (1,000 μ g/ml), bacterial endotoxin (LPS) (1 μ g/ml), oligo DNA with CpG motif (CpG-ODN) (5 μ g/ml), or oligo DNA with GpC motif (GpC-ODN) (5 μ g/ml) at 37 °C for 0, 10, 20, 30, and 40 min. One μ g protein for detection of p38 and Erk 1/2, and 4 μ g protein for JNK detection were separated for on SDS-11% polyacrylamide gel and electroblotted to PVDF membrane. Total MAPK and dual phosphorylated MAPK were detected with specific antibodies. Pictures from one experiment representative of three are shown.

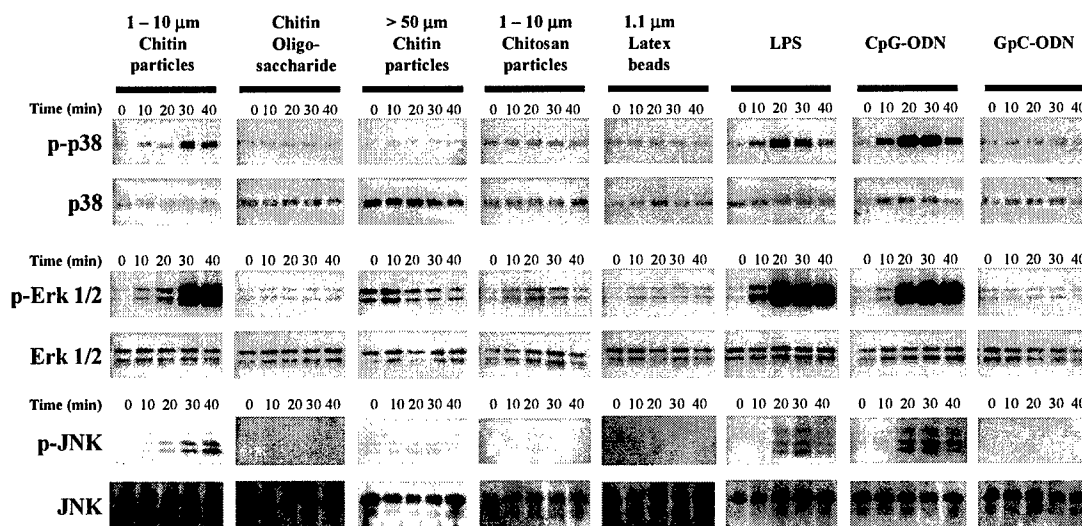
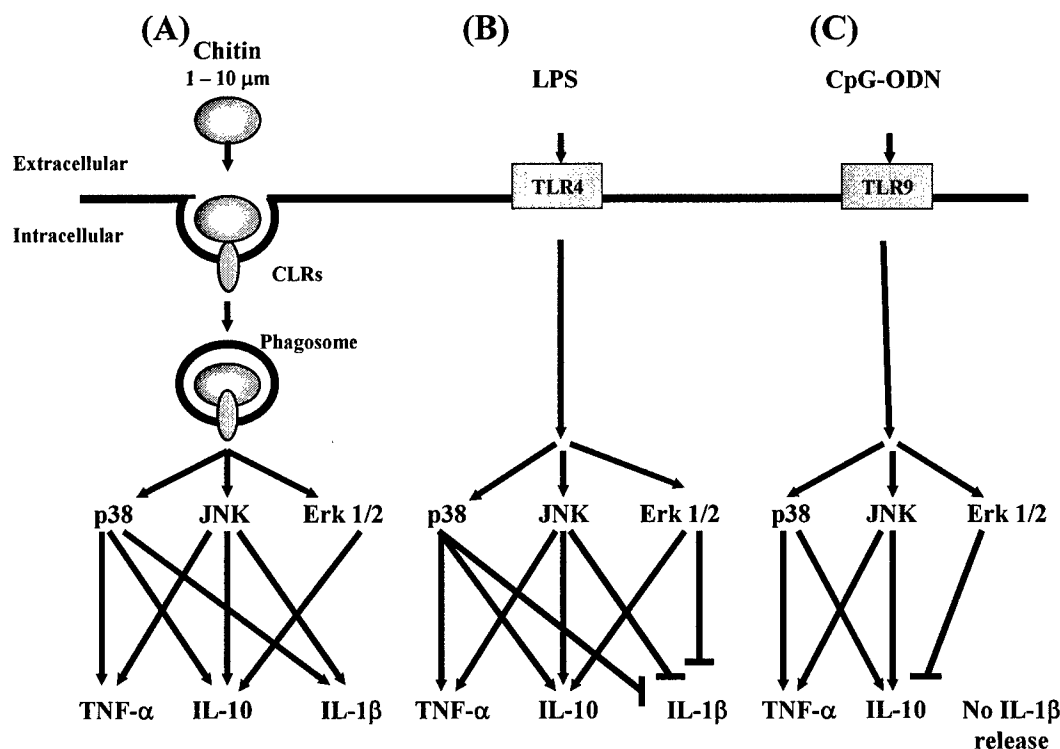


Figure 2. Schematic representation of chitin particle-induced MAPK activation regulating TNF- α , IL-10, and IL-1 β production. Scheme of chitin particle-induced MAPK activation was proposed from this study. CLRs (C-type lectin-like receptors) located on M ϕ plasma membrane interact with chitin. As comparison controls, signals that are induced by LPS and CpG-ODN are mediated by TLR4 and TLR9, respectively.



Summary: When mouse macrophages (MØ) phagocytose *N*-acetyl-β-D-glucosamine polymer (chitin) particles, they produce cytokines including TNF-α, IL-10, and IL-1β. We determined whether 1 – 10 μm chitin particles activate mitogen-activated protein kinase (MAPK) families that regulate the production of TNF-α, IL-10, and IL-1β. We used RAW264.7 MØ-like cells, in which p38 MAPK (p38), c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (Erk) 1/2 are activated by bacterial endotoxin (LPS) and oligonucleotides with CpG motif (CpG-ODN). We found that 1 - 10 μm chitin particles also activated p38, JNK, and Erk 1/2 with production of large amounts of TNF-α and IL-1β and a lesser amount of IL-10. Soluble chitin, larger sizes of chitin (>50 μm), chitosan (de-acetylated chitin) particles (1 - 10 μm), or latex beads (1.1 μm) failed to induce either MAPK activation or cytokine production. Inhibitory studies with SB203580, PD98059, and SP600125 showed that both p38 and JNK pathways are involved in chitin-induced IL-1β and TNF-α production whereas all three pathways are essential for IL-10 production. The contribution of each MAPK family for IL-1β and IL-10 production was distinct for each agonist. We conclude that MAPK activation contributes to MØ cytokine production when MØ phagocytose micro-particles through recognition of *N*-acetyl-β-D-glucosamine residues.

KEY RESEARCH ACCOMPLISHMENTS:

1. Further definition of cellular mechanisms underlying phagocytosis of chitin particles and production of anti-inflammatory cytokines by macrophages.
2. Characterization of splenic PGE₂-releasing macrophages that potentially reduce Th1 adjuvant activity, including discordance between PGE₂ production and COX-2 expression.

REPORTABLE OUTCOMES:

Manuscript in press.

“Differential effects of IL-10 on prostaglandin H synthase-2 expression and prostaglandin E₂ biosynthesis between spleen and bone marrow macrophages,” by Y Shibata, A Nishiyama, H Ohata, J Gabbard, QN Myrvik, RA Henriksen, J. Leukocyte Biol (in press). Task 2 and 3 (Appendix I)

Manuscript in preparation.

“Marrow-derived splenic macrophages expressing PGHS-2 may contribute to increased PGE₂ production in BCG-immunized mice,” by Y. Shibata, M. Smith, A. Nishiyama, H. Ohata, J. Gabbard, Q. N. Myrvik, R. A. Henriksen. Task 2a (Appendix II)

“Mitogen-activated protein kinase activation is associated with TNF-α, IL-10, and IL-1β production by RAW264.7 cells phagocytosing chitin particles,” by A. Nishiyama, J. Gabbard, H. Ohata, R.A. Henriksen, Q. N. Myrvik, and Y. Shibata. Task 2a and Task 3 (Appendix III).

Presentations

“Marrow-derived splenic macrophages expressing Cox-2 may contribute to increased PGE₂ production in BCG-immunized mice,” by Y Shibata, M Smith, QN Myrvik, H Ohata, A Nishiyama, RA Henriksen, the 2004 Experimental Biology, 4/17 – 4/21/04, Washington DC. Task 2a (Appendix IV)

“Mycobacteria-induced osteoclastogenesis and PGE₂-releasing macrophage formation in the mouse spleen,” by H Ohata, A Nishiyama, RA Henriksen, QN Myrvik, Y Shibata, the 2004 Experimental Biology, 4/17-4/21/04, Washington DC. Task 2a, (Appendix V)

“Mechanism of phagocytosed particle-induced IL-12 production in macrophage,” by A Nishiyama, H Ohata, RA Henriksen, QN Myrvik, Y Shibata, the 2004 Experimental Biology, 4/17-4/21/04, Washington DC. Tasks 1a, 3a and 3b, (Appendix VI)

“Oral administration of N-acetyl-D-glucosamine polymer particles down-regulates allergic responses,” by Y Shibata, MR Van Scott, A Nishiyama, H Ohata, QN Myrvik, the CDMRP’s Military Health Research Forum (MHRF), 4/25-4/28/04, 2004, San Juan, Puerto Rico. Tasks 1a and 1b, (Appendix VII).

Employment

Dr. Hiroyoshi Ohata, Postdoctoral Research Associate, resigned October 2004. Two Postdoctoral Research Associates, Shoutaro Tsuji, Ph.D. and Makiko Y. Tsuji, DVM, Ph.D., will join the laboratory and conduct this project starting in April 2005.

CONCLUSIONS:

We originally found (2,3), and other groups (4,5) confirmed, that phagocytosable chitin particles induce the activation of macrophages (MØ) leading to innate immunity characterized by production of Th1 cytokines including IL-12, an anti-inflammatory cytokine, but not the Th2 cytokine IL-10, a pro-inflammatory cytokine. Chitin-induced innate immunity not only results in microbicidal MØ formation but also, when mixed with water-soluble allergens, induces acquired Th1 lymphocyte responses (1,6,7) and reduces Th2 responses (1,4). Chitin particles bind to C-type lectin like receptors (CLR) including mannose receptors on MØ plasma membranes and CLR/particle complexes are internalized. The phagocytic process activates mitogen-activated protein kinases (MAPK), which are obligatory mediators of cytokine production. However, chitosan (deacetylated chitin) was phagocytosed in a mannose receptor-independent manner and failed to induce either MAPK activation or cytokine production. Small soluble chitin polymer also failed to induce either event.

Mycobacterial components (HK-BCG, DNA), Th1 adjuvants, have been used for down-regulation of allergic asthma (8-10). These agents frequently induce high levels of MØ IL-10 (Appendix III), formation of PGE₂-releasing splenic MØ (PGE₂-MØ) (7,11), and Th1-to-Th2 shifts of immune responses (12,13-17), all potentially promoting allergic responses. The expression of IL-10 enhances airway hyperresponsiveness (18). PGE₂, furthermore, enhances Th2 immune responses while inhibiting Th1 responses. We found that PGE₂-MØ are derived from radiosensitive bone marrow, although maturation requires the splenic hemopoietic environment. *Mycobacterium bovis* BCG components including oligonucleotides with unmethylated CpG motifs (ODN-CpG) are known to induce IL-10 production and PGE₂-MØ formation as well as Th1 cytokine production. In sharp contrast, chitin as a Th1 adjuvant has been established to inhibit both IL-10 production and the formation of PGE₂-MØ. Thus chitin may be the most potent Th1 adjuvant presently available and is an attractive immunomodulator for allergic asthma.

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APPENDICES:

Appendix I: Y Shibata, A Nishiyama, H Ohata, J Gabbard, QN Myrvik, RA Henriksen, Differential effects of IL-10 on prostaglandin H synthase-2 expression and prostaglandin E₂ biosynthesis between spleen and bone marrow macrophages, J. Leukocyte Biol (in press).

Appendix II: Y Shibata, M Smith, A Nishiyama, H Ohata, J Gabbard, QN Myrvik, RA Henriksen, Marrow-derived splenic macrophages expressing PGHS-2 may contribute to increased PGE₂ production in BCG-immunized mice, Manuscript in preparation.

Appendix III. A Nishiyama, J Gabbard, H Ohata, RA Henriksen, QN Myrvik, Y Shibata, Mitogen-activated protein kinase activation is associated with TNF- α , IL-10, and IL-1 β production by RAW264.7 cells phagocytosing chitin particles, Manuscript in preparation.

Appendix IV: Y Shibata, M Smith, QN Myrvik, H Ohata, A Nishiyama, RA Henriksen, Marrow-derived splenic macrophages expressing Cox-2 may contribute to increased PGE₂ production in BCG-immunized mice," Abstract was presented at the 2004 Experimental Biology, 4/17 – 4/21/04, Washington DC.

Appendix V: H Ohata, A Nishiyama, RA Henriksen, QN Myrvik, Y Shibata, Mycobacteria-induced osteoclastogenesis and PGE₂-releasing macrophage formation in the mouse spleen, Abstract was presented at the 2004 Experimental Biology, 4/17-4/21/04, Washington DC.

Appendix VI: A Nishiyama, H Ohata, RA Henriksen, QN Myrvik, Y Shibata, Mechanism of phagocytosed particle-induced IL-12 production in macrophage, Abstract was presented at the 2004 Experimental Biology, 4/17-4/21/04, Washington DC.

Appendix VII: Y Shibata, MR Van Scott, A Nishiyama, H Ohata, QN Myrvik, Oral administration of N-acetyl-D-glucosamine polymer particles down-regulates allergic responses, Abstract was presented at the CDMRP's Military Health Research Forum (MHRF), 4/25-4/28/04, 2004, San Juan, Puerto Rico.

Appendix I

Differential effects of IL-10 on prostaglandin H synthase-2 expression and prostaglandin E₂ biosynthesis between spleen and bone marrow macrophages

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Running title: Heterogeneity of PGE₂-releasing macrophages

ABSTRACT

Different Comparisons of populations or compartments of mononuclear phagocytes (MØ) show considerable diversity of cellular function including PGE₂ biosynthesis. Certain Treatments with bacterial components can enhance PGE₂ biosynthesis differentially in selected populations indicating modulation is also important determinant of MØ diversity. IL-10 is proposed to inhibit the modulation of PGE₂ biosynthesis through by down-regulating prostaglandin G/H synthase-2 (PGHS-2)- expression in some MØ. To assess whether IL-10 regulates PGE₂ biosynthesis and PGHS-2 expression, in splenic and bone marrow MØ, these MØ were isolated from IL-10- deficient (IL-10^{-/-}), C57Bl/6 (wild type [WT] control) and Balb/c (comparison control) mice and treated *in vitro* with LPS and/or IFN γ as a model of bacterial inflammation. We evaluated the levels of PGE₂ release and PGHS-1 and 2 protein expressions. LPS-induced PGHS-2 expression in IL-10^{-/-} spleen MØ was similar to that from for splenic MØ isolated from Balb/c and C57Bl/6 the three strains of mice. However, PGE₂ levels released by LPS-treated splenic MØ exhibited wasslight but significantly higher in IL-10^{-/-} and Balb/c than those in WT miccells. In the presence of LPS and IFN γ , PGHS-2 expression and PGE₂ release by both IL-10^{-/-} and Balb/c mice splenic MØ were enhanced compared to stimulation with LPS alone or IFN γ alone. However, in C57Bl/6 WT mice, there was no significant increase in PGE₂ release from WT splenic MØ by treated with LPS plus IFN γ despite a synergistic increased in PGHS-2 expression.. PGHS-1 protein levels were not affected by LPS stimulation in either WT or IL-10^{-/-} mice. In sharp contrast, PGHS-2 expression and PGE₂ release by bone marrow MØ were greatly enhanced in IL-10^{-/-} miccells compared to control cells. Our results indicate various degrees of that IL-10-mediate regulation of MØ PGE₂ biosynthesis and PGHS-2 expression that are compartment-dependent, and that PGE₂ production is not directly linked to PGHS-2 levels. Furthermore, our findings emphasize strain-specific differences between C57Bl/6 and Balb/c mice with Balb/c appearing more similar to the IL-10^{-/-} than to the C57Bl/6 with respect to prostanoid production.

INTRODUCTION

Mononuclear phagocytes (MØ) are a major source of prostaglandin E₂ (PGE₂), an arachidonic acid (AA) metabolite, that regulates the immune response, hematopoiesis, inflammation, tissue injury and repair, and bone resorption. The regulation of these events may be closely related to the regulation of PGE₂ release by MØ (1). The effective *in vivo* expression of these responses, furthermore, may depend on the presence of an adequate number of MØ with appropriate functions in specialized locations (2,3). We are interested in the lymphoid tissue of spleen where PGE₂-releasing MØ (PGE₂-MØ) and immune lymphocytes interact in chronic inflammatory diseases including mycobacterial infections. PGE₂ inhibits the production of Th1 cytokines such as IL-2, IL-12 and IFN γ (4). In contrast, PGE₂, depending on stimulatory conditions, either has no effect or enhances production of Th2 cytokines, such as IL-4, IL-5 and IL-10 (4,5). Therefore, increases in splenic PGE₂-MØ may underlie the Th1-to-Th2 shift of immune responses that are major pathogenic events in chronic inflammatory diseases. More studies are needed to elucidate the mechanism of splenic PGE₂ production and the mechanism of splenic PGE₂-MØ formation.

Normal splenic MØ, unlike peritoneal and bone marrow MØ or monocytes, produce relatively low levels of PGE₂ biosynthesis (<5 ng of PGE₂/5 x 10⁶ MØ/ml) (6). However, previous studies (7) have shown that an *in vivo* response to chronic inflammatory conditions including mycobacterial infections is manifested by the emergence of splenic MØ with the capacity to form large amounts of PGE₂ (>50 ng/ml). The mechanisms for splenic PGE₂-MØ formation appears to be complex. Our studies have shown that their formation is dependent on radiosensitive bone marrow cells, which may supply precursors of splenic PGE₂-MØ (2). It is likely that the precursors migrate and localize to the spleen where mature forms of PGE₂-MØ are established (6). Alternatively, an inflammatory cytokine "milieu" may directly up-modulate PGE₂ biosynthesis of by splenic MØ (8,9).

PGE₂-MØ metabolize endogenous arachidonic acid (AA) to PGE₂ through cyclooxygenase (PGHS, EC 1.4.99.1), a rate-limiting enzymes for prostaglandins and thromboxane production. Two major isoforms of PGHS synthase exist: PGHS-1, a constitutive form; and PGHS-2, an inducible form that is rapidly up-regulated

in response to LPS and pro-inflammatory cytokines (10-12). The formation of splenic PGE₂-MØ is expected, therefore, to be dependent on the level and activity of PGHS-2 induced (8).

Cytokines, which are present in both pro- and anti-inflammatory conditions, modulate PGE₂ synthesis by MØ cell lines (13), MØ freshly isolated from the peritonea, and lungs or those derived from monocytes (8,11,12,14). TNF α (15), IL-1 α (15,16), and IFN γ (17,18) have been demonstrated to induce PGHS-2 expression, whereas IL-4 (19), IL-13 (20,21), IL-10 (22), and TGF- β (23) can inhibit PGHS-2 induction. The question addressed in these studies is whether the level of PGHS-2 expression under selected inflammatory conditions is dependent on IL-10 and directly correlated to with PGE₂ biosynthesis in splenic MØ.

IL-10 is a MØ deactivator, blocking LPS-induced synthesis of TNF α , IL-1 β , IL-6, IL-8, IL-12 and GM-CSF by human monocytes (24), mouse peritoneal MØ (25) and mouse splenic MØ (26). It has been reported that IL-10 can inhibit PGHS-2 induction *in vitro* in human monocytes and neutrophils (19,22). Recently, Berg et al (8) demonstrated that LPS induces normal mouse murine spleen MØ to express PGHS-2 and synthesize PGE₂ biosynthesis that are down-regulated by endogenous IL-10. Our results with IL-10^{-/-} mice MØ show that in the presence of LPS, IL-10 down-regulates PGE₂ synthesis without an equivalent effect on PGHS-2 expression.

MATERIALS AND METHODS

Animals. Healthy 8- to 12-wk-old IL-10^{-/-} female mice on C57Bl/6 background were obtained from Jackson Lab (Bar Harbor, ME). Wild type (WT) C57Bl/6 and Balb/c female mice were obtained from Jackson Lab. All mice were maintained in microisolator cages under specific pathogen-free conditions at in the animal care facility at East Carolina University. and Florida Atlantic University.

Reagents. LPS from *Escherichia coli* (serotype 0111:B4, phenol extraction, L-2630) and mouse recombinant IFN γ (I-4777) were obtained from Sigma, (St. Louis, MO) and reconstituted in pyrogen-free saline. Mouse rRecombinant murine IL-10 was obtained from PharMingen (San Diego, CA). A23187 (Sigma) was dissolved in DMSO at 1 mg/ml. AA (Sigma) was dissolved in 100% ethanol at 1 mg/ml. Rabbit polyclonal anti-murine PGHS-1 and rabbit polyclonal anti-PGHS-2 were obtained from Cayman Chemicals (Ann Arbor, MI).

MØ preparations from spleen and bone marrow. Spleens in each group of mice (at least 5 mice per group) were isolated and pooled. Excised spleens were minced with scissors and digested with 50 U/ml collagenase D (C-2139, Sigma) at 50 U/ml in RPMI 1640 plus 10% FBS at 37°C for 60 min in and filtered through a 100- μ m mesh. After washing digested cells with RPMI 1640 in the presence of 100 μ g/ml DNase at 100 μ g/ml (DN-25, Sigma), cells were suspended in RPMI 1640 plus 10% FBS. Bone marrow cells were isolated by flushing the marrow cavities of the femurs with ice-cold RPMI 1640 medium and gently refluxing the expelled cell plug with a Pasteur pipette to form a single cells suspension. To enrich MØ fraction (2,6), spleen cell and bone marrow cell suspensions were layered over top of a discontinuous Percoll gradient (35/60%, Sigma). Following centrifugation (800xg for 30 min at 22°C), cells in the layer between 35% and 60% Percoll were collected, washed and suspended in RPMI 1640 medium plus 10% FBS. These cells were plated at approximately 3 – 5 x 10⁶ cells/ml per 35 mm culture dish (Falcon, Oxnard, CA) and incubated at 37°C in 5% CO₂ in air. After 2 hr incubation, the cells were washed with Ca²⁺ and Mg²⁺-free 0.15 M phosphate-buffered saline (PBS) for removal of the nonadherent cells. Following placing the dish and held on ice for 30 minutes, the adherent cells were harvested by scraping and washed twice with serum-free RPMI 1640. Viability was >90% (trypan blue exclusion). Adherent spleen cells were >85% MØ; and adherent marrow cells were >92% MØ, both estimated with by phagocytosis of IgG-opsonized sheep red cells (2) and/or cytometrically following staining with anti-Mac-1 (27).

Cell culture protocols. Splenic MØ and bone marrow MØ were isolated above and cultured at 5 x 10⁶ cells/ml in RPMI 1640 supplemented with 5 % FBS, penicillin (100 U/ml), streptomycin (100 U/ml) and amphotericin B

(2.5 µg/ml) in 12 x 75 mm culture tubes (0.5 ml/tube, Costar, Corning, NY). Cells were incubated in medium alone or medium supplemented with LPS (10 µg/ml), IFN γ (10 ng/ml) or LPS plus IFN γ mixture (both at same final concentrations). In some experiments where indicated, exogenous IL-10 at 0.1, 1 or 10 ng/ml was added to the culture. After 24 hrs, the cells were washed with cold saline and treated with a lysis buffer as described below. To elicit PGE $_2$ release, the cultured cells were washed, suspended in serum-free RPMI 1640, and incubated with agonists (A23187, AA, or LPS) at 37°C. Supernatants from triplicate cultures were harvested after 2 hr and stored at -70°C before analysis for PGE $_2$.

Quantification of PGE $_2$ and TxB $_2$. PGE $_2$ and TxB $_2$ levels in tissue culture supernatants were determined using enzyme immunoassay kits (Cayman Chemicals, Ann Arbor MI) according to the manufacturer's instructions.

Western blotting. Splenic M ϕ and bone marrow M ϕ were cultured as described above, harvested and washed 3 times with cold saline. Protein was isolated from washed cells by resuspending in lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, Sigma Protease Inhibitor Cocktail [1:500, P8340, Sigma], 1 % Nonidet P-40, and 1% sodium deoxycholate). Debris was eliminated by centrifugation (5 min, 1000 x g). Protein concentration was measured using a commercial reagent based on bicinchoninic acid staining (Pierce, Rockford, IL) using BSA as standard. Equal amounts of cellular protein were loaded onto SDS-polyacrylamide gel and separated by electrophoresis (200 V for 45 min). Proteins were then transferred to PVDF membrane (100 V for 1 h), and the membrane was blocked with 5% nonfat dry milk. The membrane was then incubated with antibody (anti-PGHS-1, 1:1000; anti-PGHS-2, 1:4000) overnight at 4°C. Following incubation with peroxidase conjugated donkey anti rabbit IgG (Jackson ImmunoResearch, West Grove, PA), proteins were detected by chemiluminescence (ECL, Amersham, Piscataway, NJ) per following the manufacturer's instructions.

Statistics. Data from this project were analyzed by one way analysis of variance. For cell culture studies, tissues isolated from at least 5 mice were pooled unless indicated; these cells were cultured in at least triplicate in each group. *P* value of less than 0.05 is considered statistically significant.

RESULTS

PGE $_2$ release by splenic M ϕ isolated from IL-10 $^{-/-}$, C57Bl/6 (WT), and Balb/c mice. Normal murine splenic M ϕ release PGE $_2$ at minimum levels compared to bone marrow M ϕ (3,6). To determine whether endogenous IL-10 regulates PGE $_2$ released by splenic M ϕ in response to the proinflammatory stimuli LPS and/or IFN γ , splenic M ϕ were isolated from C57Bl/6 (WT), and IL-10 $^{-/-}$ mice. Recently, Kuroda and Yamashita (9) found differential release of PGE $_2$ by splenic M ϕ isolated from C57Bl/6 WT and Balb/c mice when stimulated by LPS *in vitro*. We, therefore, employed splenic M ϕ isolated from Balb/c mice as comparison controls. The M ϕ were primed with LPS (10 µg/ml), IFN γ (10 ng/ml), LPS combined with IFN γ (at same final concentrations), or medium alone for 24 hr, followed by the elicitation of PGE $_2$ release with 1 µM calcium ionophore A23187 for 2 hrs. As shown in Figure 1, PGE $_2$ release was slightly but significantly enhanced in Balb/c and IL-10 $^{-/-}$ mice compared to WT when splenic M ϕ were primed with LPS in Balb/c and IL-10 $^{-/-}$ mice. This enhancement was completely diminished/eliminated when 1 µM NS-398 at 1 µM was added in to the LPS-primed cultures (data not shown). Although IFN γ did not prime PGE $_2$ biosynthesis by itself, it showed a synergistic effect when mixed with in the presence of LPS. Such priming effects of LPS and IFN γ were not observed in splenic M ϕ from C57Bl/6 WT mice (Figure 1).

PGE $_2$ release by bone marrow M ϕ isolated from IL-10 $^{-/-}$, C57Bl/6 (WT), and Balb/c mice. When elicited with 1 µM A23187, bone marrow M ϕ from Balb/c, C57Bl/6 WT or IL-10 $^{-/-}$ mice released 10-fold more PGE $_2$ than normal splenic M ϕ (Figure 1). For the control cells, Balb/c or WT, PGE $_2$ release was not increased when bone marrow M ϕ from Balb/c and C57Bl/6 mice were primed with LPS or IFN γ alone., although LPS plus IFN γ significantly enhanced PGE $_2$ release in Balb/c but not C57Bl/6 mice. In sharp contrast, bone marrow M ϕ from IL-10 $^{-/-}$ mice showed 10-fold increases in PGE $_2$ release in response to LPS alone, without further

enhancement by the mixture of IFN γ and LPS (Figure 1). Elicitation with 1 μ g/ml AA at 1 μ g/ml also showed similar profiles of PGE $_2$ release but at significantly lower amount levels among the treatment groups than those elicited by A23187 (Table 1). However, the elicitation by 1 μ g/ml of LPS did not significantly enhance PGE $_2$ production compared to that by buffer alone in any of the primed groups (data not shown Table 1). This may be a consequence of the experimental conditions which resulted in the cells becoming refractory to the lower concentration of LPS used for elicitation after the long period of priming at higher LPS.

Thromboxane release by M ϕ . To determine whether the effects observed for PGE $_2$ production were general for all prostanoids, thromboxane B $_2$ levels were also determined in M ϕ supernatants. For splenic M ϕ pre-treated with LPS and/or IFN γ , there was no increase of in thromboxane (TxB $_2$) release following by splenic M ϕ isolated from C57Bl/6 and Balb/c mice that were pre-treatment ed with LPS and/or IFN γ compared with medium alone. Thromboxane production was not enhanced in IL-10 $^{-/-}$ mice (Figure 2). Furthermore, as shown in Figure 2, bone marrow M ϕ from C57Bl/6 and Balb/c mice released TxB $_2$ at significantly higher levels compared to those from splenic M ϕ similarly to what was observed for PGE $_2$ (Figure 1). However, priming with LPS or LPS combined with and IFN γ did not result in increases in TxB $_2$ release, but rather the tended to suppression of thromboxane production (Figure 2). Our results clearly further demonstrated that endogenous IL-10 had no significant effect on the thromboxane production by splenic or bone marrow M ϕ (Figure 2).

Effect of endogenous IL-10 on protein expression of PGHS-1 and PGHS-2. To determine whether endogenous IL-10 alters the level of protein expression of PGHS-1 and PGHS-2, Western blot analyses employing specific antibodies against for PGHS-1 and PGHS-2 were performed. Untreated splenic M ϕ isolated from Balb/c, C57Bl/6 WT and IL-10 $^{-/-}$ mice showed PGHS-1 expression and the levels were relatively stable following the treatment s with IFN γ and/or LPS (Figure 3). On the other hand, levels of PGHS-2 protein in untreated splenic M ϕ isolated from Balb/c, C57Bl/6 and IL-10 $^{-/-}$ mice were undetectable. Stimulation of splenic M ϕ with LPS resulted in marked increases in PGHS-2 protein at comparable levels among the three strains of mice. The mixture of LPS and IFN γ further enhanced PGHS-2 levels in all strains, although the PGHS-2 proteins were slightly more increase d was greater in Balb/c than in C57Bl/6 WT and IL-10 $^{-/-}$ (Figure 3).

As for spleen M ϕ , PGHS-2 was not detected in untreated bone marrow M ϕ . Figure 4 shows that bBone marrow M ϕ from Balb/c and C57Bl/6 WT mice also showed increaseds in PGHS-2 levels in response to LPS (Figure 4). These levels were further enhanced whenby LPS was combined with IFN γ (Figure 4). In IL-10 $^{-/-}$ mice, PGHS-2 levels that were induced by LPS or LPS plus IFN γ were significantly further enhanced in IL-10 $^{-/-}$ mice compared to C57Bl/6 WT orand Balb/c mice (Figure 4). Bone marrow M ϕ PGHS-1 expression was unchanged among the treatment groups as well as among the three mouse strains (Figure 4).

Effects of exogenous IL-10 on PGHS-2 expression and PGE $_2$ synthesis. To determine whether the effects observed in IL-10 $^{-/-}$ cells could be reversed by addition of exogenous IL-10, When splenic and bone marrow M ϕ were isolated from WT mice and primed with LPS plus IFN γ . , In the absence of added IL-10, the culture supernatants of splenic and bone marrow M ϕ contained IL-10 at 121 and 267 pg/ml/5 x 10 6 M ϕ , respectively., was produced. ENeither splenic norand bone marrow M ϕ that were isolated from IL-10 $^{-/-}$ and primed with LPS plus IFN γ did not produced IL-10 (<25 pg/ml). To further confirm the effects of IL-10, Exogenous IL-10 at 0.1, 1 or 10 ng/ml was added to the IL-10 $^{-/-}$ M ϕ cultures prepared from IL-10 $^{-/-}$ mice. I In splenic M ϕ , the highest dose of IL-10 (10 ng/ml), but not 0.1 or 1 ng/ml of IL-10, inhibited both PGHS-2 protein expression and PGE $_2$ synthesis. In sharp contrast, for bone marrow M ϕ from IL-10 $^{-/-}$ mice, in sharp contrast, IL-10 at these concentrations at 0.1, 1 and 10 ng/ml inhibited PGE $_2$ synthesis and PGHS-2 expression in a dose dependent manner (Figure 6). As shown above (Figure 1), when WT splenic or marrow M ϕ from WT mice were primed with LPS plus IFN γ , PGE $_2$ synthesis was not increased. Exogenous IL-10 did not inhibit PGE $_2$ synthesis by either splenic or marrow M ϕ (Figures 5 and 6)., although IL-10 at 10 ng/ml was inhibitory for PGHS-2 expression in these M ϕ . Finally, exogenous as well as like endogenous IL-10 did not inhibit PGHS-1 protein expression by splenic and marrow M ϕ (Figures 5 and 3-6). These Taken together, our results suggest indicate that the inhibitory effects of IL-10 on PGHS-2-mediated PGE $_2$ synthesis in inflammation are significant in both

splenic and marrow MØ, but the magnitude of the IL-10 effect on PGHS-2-mediated PGE₂ synthesis was greater in bone marrow MØ than in splenic MØ.

DISCUSSION

Regulation of PGE₂ Production. Mononuclear phagocytes (MØ) are a major source of PGE₂ a key mediator regulating inflammatory responses (28-33). Heterogeneity of PGE₂ biosynthesis among MØ populations has been well documented (3,34). Normal splenic MØ express relatively low levels of PGE₂ (<5 ng of PGE₂/5 x 10⁶ MØ/ml). However, 7 to 14 days after C57Bl/6 and CBA/J mice are challenged with mycobacteria or related components, splenic MØ show a 10-fold increase in PGE₂ release (>50 ng/ml) (2,3,6). Exact mechanisms for splenic PGE₂-MØ formation are still unknown. The present *in vitro* studies examined PGE₂ biosynthesis in MØ treated with LPS and/or IFN γ , proinflammatory agents, used to simulate conditions present in bacterial inflammation. We have investigated the role of IL-10 in regulating *in vitro* PGE₂ production by splenic and bone marrow MØ with cells derived from IL-10^{-/-} and two control strains of mice. Because the IL-10^{-/-} mice are on a C57Bl/6 (WT) background, this species is the most relevant control for determining the effects of IL-10. When splenic MØ were primed with LPS, there was a significant increase in PGE₂ in the IL-10^{-/-} cells as well as in Balb/c cells. When LPS primed IL-10^{-/-} splenic MØ are compared to WT cells, the results indicate that endogenous IL-10 causes significant suppression of PGE₂ production to approximately the level seen in unprimed cells (Figure 1). We found that IL-10 also contributes to the down-regulation of PGE₂ biosynthesis by LPS-primed bone marrow MØ. In this case, the difference in PGE₂ production between WT and Balb/c was less pronounced than for the corresponding splenic cells. Interestingly, PGE₂ synthesis is much greater in bone marrow MØ than in splenic MØ as is the magnitude of the IL-10 effect (Figure 1). Our *in vitro* studies indicate that IL-10 does not regulate thromboxane production (Figure 2). The results for Balb/c and WT mice also indicate a genetically determined difference in the murine inflammatory response, consistent with an earlier report (35).

LPS priming of MØ enhancement of PGHS-2 is independent of IL-10. We further analyzed the effects of IL-10 on PGHS-2 expression in relation to PGE₂ biosynthesis in both splenic and marrow MØ. Our studies demonstrate that LPS induces PGHS-2 protein expression by IL-10^{-/-} splenic MØ with a profile similar to that seen in splenic MØ from WT or Balb/c mice, suggesting that this response is not highly dependent on IL-10. Neither the inflammatory mediators nor IL-10 appear to exert any regulatory effect on PGHS-1 protein expression, similar to previous reports indicating that PGHS-1 expression is generally constitutive rather than inducible (8). Previous studies of PGE₂ production by rat alveolar MØ in response to LPS, inflammatory cytokines, and/or mitogens concluded that PGE₂ production is significantly associated with an increase in PGHS-2 protein levels (12). Examination of our data (Figures 1, 3, 4), indicates that in IL-10^{-/-} mice there appears to be a dependence of PGE₂ production on PGHS-2 levels as previously reported. This is particularly evident in bone marrow MØ where the magnitude of PGE₂ production is greatest. However for WT cells, the levels of PGE₂ synthesis do not correspond with PGHS-2 levels. Increased PGE₂ production does not appear to occur in the absence of increased expression of PGHS-2, although increased PGHS-2 expression does not necessarily lead to increased PGE₂ production. Therefore, factors other than PGHS-2 expression, must contribute to the IL-10 dependent regulation of PGE₂ biosynthesis.

In our previous studies (2,3,6,36) of C57Bl/6 and CBA/J mice immunized with BCG or *C. parvum*, establishment of high PGE₂-releasing splenic MØ required 7 – 14 days (>50 ng of PGE₂/5 x 10⁶ MØ). These MØ expressed PGHS-1 and PGHS-2, although the contribution of endogenous IL-10 to PGHS-2-mediated PGE₂ synthesis by the MØ was unclear. Interestingly, splenic MØ isolated one day after immunization showed relatively high PGHS-2 expression in an IL-10-independent manner (Shibata et al, unpublished observation). The expression of PGHS-2 was not observed on day 2 after BCG immunization. Unlike splenic MØ isolated 7 – 14 days after immunization, however, high PGHS-2 levels on day 1 were not associated with high PGE₂ synthesis since splenic MØ isolated from days 1, 2 and 3 after BCG immunization showed no increase in PGE₂

release (<5 ng/ml) (2,6). This *in vivo* observation suggests that there is also a temporal component to the development of PGE₂ production.

Contribution of IFN γ . Our results indicate that in the presence of LPS, PGE₂ production in both IL-10^{-/-} and Balb/c splenic M ϕ is enhanced by addition of IFN γ . In WT mice, there was no significant increase in PGE₂ release in the presence of LPS or IFN γ either singly or combined (Figure 1) despite the increase in PGHS-2 expression in the presence of LPS (Figure 3). IFN γ in synergy with LPS is reported to induce PGHS-2 expression in peritoneal M ϕ (37) through the activation of NF- κ B (38). Our results with splenic and bone marrow M ϕ also suggest a synergistic effect of IFN γ on PGHS-2 expression (Figures 3 & 4).

Effect of exogenous IL-10 on PGHS-2 induction and PGE₂ synthesis. The results (Figures 5 and 6) indicate that exogenous IL-10 inhibits PGE₂ synthesis and PGHS-2 induction in IL-10^{-/-} splenic and bone marrow M ϕ confirming a regulatory role for IL-10. However, for splenic M ϕ where levels of PGE₂ synthesis are quite low, this inhibition appears to require relatively high doses of exogenous IL-10. Along with the quantitative difference in PGE₂ production, this difference in IL-10 sensitivity could also indicate a difference in regulatory mechanisms for cells of splenic and marrow origins. It may also be that exogenous IL-10 added to the media of the splenic M ϕ does not functionally or temporally reproduce IL-10 levels developed *in vivo*.

Taken together the results suggest that another effector such as PGE synthase or possibly a specific phospholipase A₂ is involved in the regulation of PGE₂ biosynthesis and that regulation of this effector is directly or indirectly dependent on IL-10. Alternatively the effects of IL-10 on PGE₂ production may be through regulation of PGHS-2 activity. One cannot rule out the contribution of PGHS-3, a PGHS-1 variant that was recently identified (39).

Role of IL-10. There are multiple possible mechanisms for IL-10 regulation of PGHS-2 synthesis (8). It is established that LPS, IL-1 β and TNF α enhance PGHS-2 mRNA stability (40), whereas exogenous IL-10 *in vitro* accelerates the degradation of PGHS-2 mRNA in human monocytes (22), consistent with our findings for addition of exogenous IL-10 to IL-10^{-/-} cells (Figures 5 and 6). In addition, previous studies (41-45) suggest that LPS-induced PGHS-2 expression and PGE₂ synthesis are associated with p38 MAPK activation in human monocytes and neutrophils. IL-10 appears to down-regulate p38 MAPK activation (45-47). p38 MAPK activation is an upstream kinase regulating NF- κ B activation in neutrophil (48), suggesting that p38 MAPK might play a role in both transcriptional and post-transcriptional regulation of the PGHS-2 gene.

Regardless of how endogenous IL-10 regulates PGE₂ synthesis, in the absence of IL-10, splenic M ϕ activated by LPS and IFN γ produce a maximum of less than 8 ng/ml of PGE₂/5 x 10⁶ M ϕ . Additional factors must contribute to the enhanced PGE₂ release by splenic PGE₂-M ϕ isolated from mice 7 – 14 days after BCG immunization (6,36). These results are consistent with our previous hypothesis that splenic PGE₂-M ϕ are derived from radiosensitive bone marrow cells (2,6). It remains to be elucidated (i) whether priming of bone marrow M ϕ *in vitro* with LPS or LPS plus IFN γ mimics the formation of splenic PGE₂-M ϕ *in vivo* and (ii) whether IL-10 down-regulates PGHS-2-mediated PGE₂ biosynthesis in splenic PGE₂-M ϕ isolated from BCG-immunized mice.

Conclusion. These *in vitro* studies comparing IL-10^{-/-} and WT cells have shown that IL-10 contributes to the down-regulation of PGE₂ biosynthesis in both splenic and marrow M ϕ previously primed with LPS. Increased PGE₂ synthesis is associated with increased PGHS-2 expression, but additional factors contribute to regulation of prostanoid production.

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Footnote

Abbreviations used in this paper: PGHS, prostaglandin G/H synthase; WT, wild-type; KO, homozygous genetic deficiency; M ϕ , macrophages; BCG, Bacillus Calmette-Guérin; PGE₂, prostaglandin E₂; AA, arachidonic acid

Table I. PGE₂ release by primed bone marrow MØ that were treated with A23187, AA or LPS

Priming (24 hr)	Elicitation (2 hr)		
	A23187 (1 µM)	AA (1 µg/ml)	LPS (1 µg/ml)
(ng PGE ₂ /5 x 10 ⁶ MØ)			
<u>IL10^{-/-} Bone marrow MØ</u>			
Medium	14.5 ± 1.5	9.8 ± 2.6	4.0 ± 0.3
IFNγ	19.4 ± 2.8	11.0 ± 3.1	5.9 ± 0.8
LPS	146.7 ± 12.1	40.9 ± 6.4	16.5 ± 4.0
IFNγ + LPS	131.3 ± 16.5	37.0 ± 5.2	15.5 ± 2.8
<u>C57Bl/6 Bone marrow MØ</u>			
Medium	21.3 ± 5.8	19.1 ± 1.6	4.2 ± 0.1
IFNγ	16.0 ± 1.7	10.4 ± 0.9	3.8 ± 1.2
LPS	23.1 ± 1.1	16.5 ± 0.5	7.3 ± 0.7
IFNγ + LPS	29.9 ± 3.5	19.1 ± 1.2	7.7 ± 0.5

Bone marrow MØ (5 x 10⁶ MØ/ml) isolated from IL-10^{-/-} and WT (C57Bl/6) mice were primed with LPS (10 µg/ml), IFNγ (10 ng/ml), LPS mixed with IFNγ (at same final concentrations), or medium alone for 24 hr. These MØ were elicited by 1 µM A23187, 1 µg/ml AA, or 1 µg/ml LPS for 2 hr. PGE₂ levels in the supernatants were measured by ELISA. Mean ± SD, n=3. Values of PGE₂ elicited with A23187 are those shown in Figure 1B. The control values of PGE₂ elicited with saline were not significantly different from those of LPS (data not shown).

FIGURE LEGENDS

Figure 1. The effects of endogenous IL-10 on PGE₂ biosynthesis in splenic and bone marrow MØ. Splenic and bone marrow MØ (5 x 10⁶ MØ/ml) isolated from IL-10^{-/-}, WT (C57Bl/6), and Balb/c mice were primed with LPS (10 µg/ml), IFNγ (10 ng/ml), LPS mixed with IFNγ (at same final concentrations), or medium alone for 24 hr. These MØ were elicited by 1 µM A23187 for 2 hrs and PGE₂ levels in supernatants were measured by ELISA. Mean ± SD, n=3. **p*<0.05, ***p*<0.01, #*p*<0.001 compared to those of WT (C57Bl/6) mice. Results are representative of two separate experiments.

Figure 2. The effects of endogenous IL-10 on TxB₂ release by splenic and bone marrow MØ. Splenic and bone marrow MØ (5 x 10⁶ MØ/ml) isolated from IL-10^{-/-} and Balb/c mice were primed with LPS (10 µg/ml), IFNγ (10 ng/ml), LPS mixed with IFNγ (at same final concentrations), or medium alone for 24 hr. These MØ were elicited by 1 µM A23187 for 2 hrs and TxB₂ levels in the supernatants were measured by ELISA. Mean ± SD, n=3. **p*<0.05, ***p*<0.01, #*p*<0.001 compared to those of medium treated cells. Results are representative of two separate experiments.

Figure 3. PGHS-1 and PGHS-2 levels in splenic MØ that were isolated from IL-10^{-/-}, WT, and Balb/c mice and primed with LPS and/or IFNγ *in vitro*. Each lane was loaded with 5 µg total protein. Results are representative three separate experiments.

Figure 4. PGHS-1 and PGHS-2 levels in bone marrow MØ that were isolated from IL-10^{-/-}, WT, and Balb/c mice and primed with LPS and/or IFNγ *in vitro*. Each lane was loaded with 5 µg total protein. Results are representative of three separate experiments.

Figure 5. The effects of exogenous IL-10 on PGHS-2 expression and PGE₂ synthesis in IL-10^{-/-} splenic MØ. Splenic MØ (5 x 10⁶ MØ/ml) isolated from IL-10^{-/-} and WT mice were primed with LPS (10 µg/ml) mixed with IFNγ (10 ng/ml) in the presence of 0 (medium), 0.01, 1 or 10 ng/ml of murine IL-10 for 24 hr. PGHS-1 and PGHS-2 levels in these MØ were determined by western blotting. Results are representative of two separate experiments. The primed MØ were elicited by 1 µM A23187 for 2 hrs and PGE₂ levels in the supernatants were measured by ELISA. Mean ± SD, n = 4. # *p*<0.001 compared to those of LPS + IFNγ alone. Similar results were obtained in a separate experiment.

Figure 6. The effects of exogenous IL-10 on PGHS-2 expression and PGE₂ synthesis in IL-10^{-/-} bone marrow MØ. Bone marrow MØ (5 x 10⁶ MØ/ml) isolated from IL-10^{-/-} and WT mice were used. All other procedures were identical to those in Figure 5. Mean ± SD, n = 3 - 4. * *p*<0.05, # *p*<0.001, ## *p*<0.001 compared to those of LPS + IFNγ alone.

Figure 1.

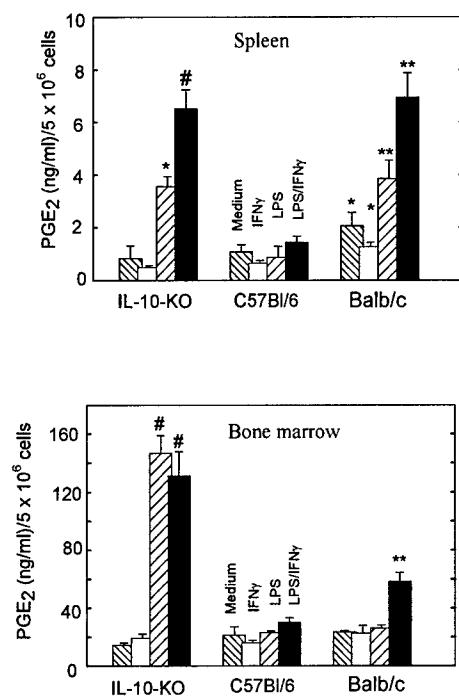


Figure 2.

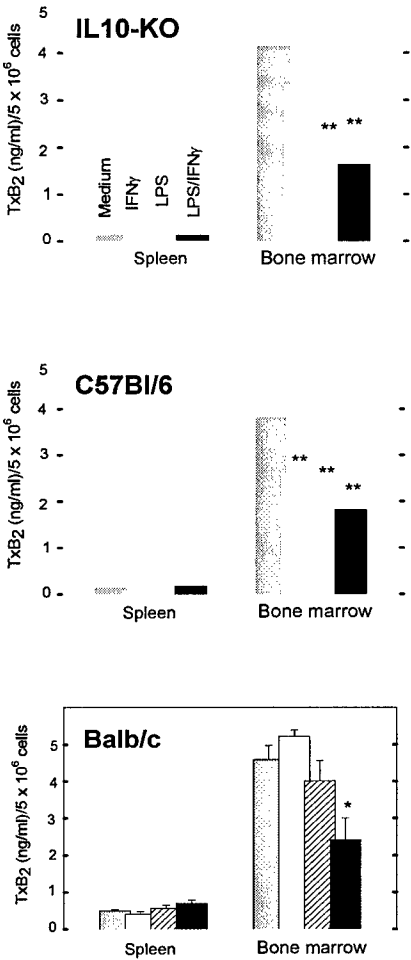


Figure 3.

Splenic Macrophages

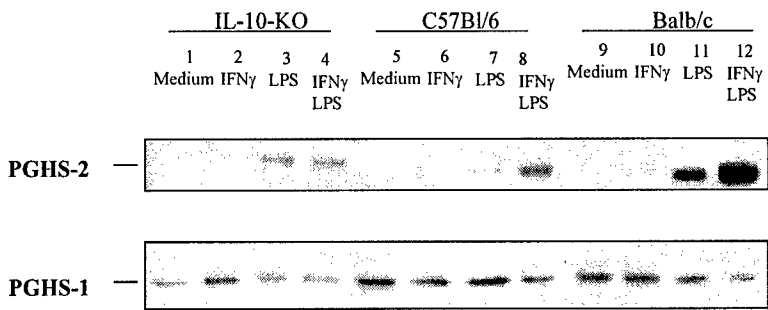


Figure 4.

Bone Marrow Macrophages

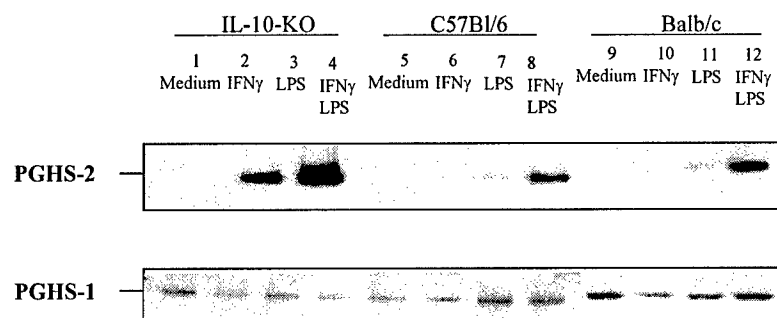


Figure 5.

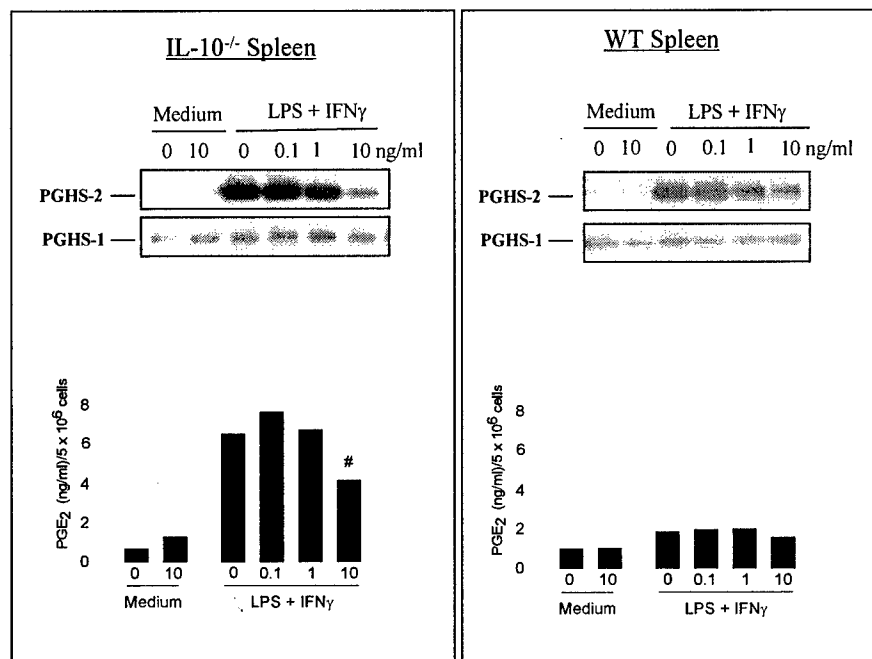
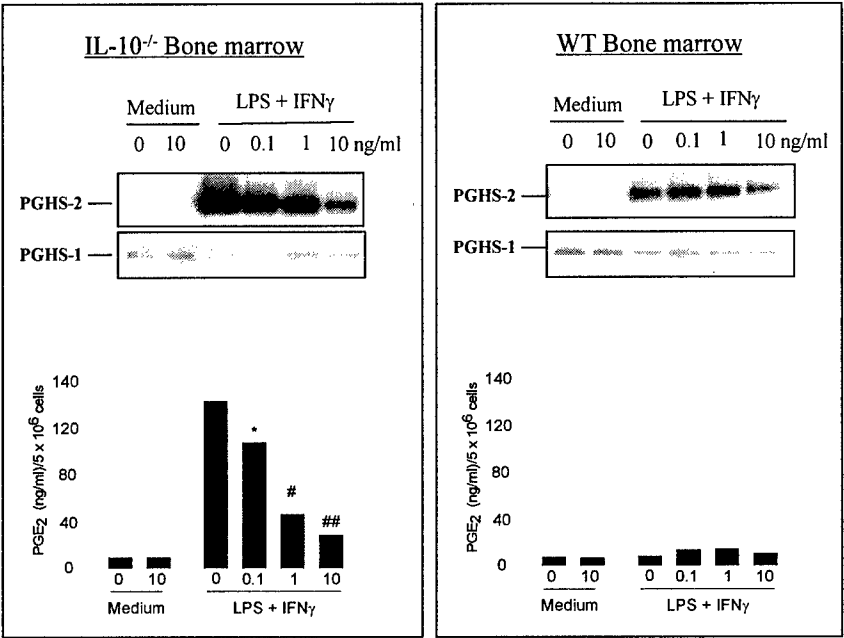


Figure 6.



Appendix II

Marrow-derived splenic macrophages expressing PGHS-2 may contribute to increased PGE₂ production in BCG-immunized mice

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Short Title: Marrow-derived splenic PGHS-2⁺ macrophages

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ABSTRACT

Previous studies indicated that, 7-14 days following exposure to mycobacterial components, *ex vivo* prostaglandin E₂ (PGE₂) release by splenic macrophages (MØ) is increased as much as 10 fold. PGE₂ induces a Th1-to-Th2 shift of immune responses against mycobacterial antigens. The formation of PGE₂-releasing splenic MØ (PGE₂-MØ) is dependent on radiosensitive bone marrow cells. Splenic PGE₂-releasing MØ (PGE₂-MØ) express both PGH synthase-1 (PGHS-1) and PGHS-2, whereas normal splenic MØ express only PGHS-1. Although splenic MØ on day 1 did not show increased PGE₂ biosynthesis, they expressed not only PGHS-1, but also a relatively high level of PGHS-2. To determine whether 14-day splenic PGE₂-MØ are derived directly from bone marrow, we established a bone marrow chimera in which C57Bl/6 recipients received bone marrow cells *iv* from green fluorescent protein (GFP)-transgenic donors. Both donors and recipients were immunized with HK-BCG simultaneously and bone marrow transfusion was performed on days 1 and 2. On day 14 after BCG immunization, a significant number of spleen cells expressed both PGHS-2 and GFP. Our results indicate that the presence of PGHS-2 is not sufficient to induce PGE₂ biosynthesis by splenic MØ and that bone marrow-derived MØ may contribute to the increased PGE₂ production observed 14 days after BCG immunization.

INTRODUCTION

Mononuclear phagocytes (MØ) metabolize endogenous arachidonic acid (AA) to prostaglandin E₂ (PGE₂) through cyclooxygenase (PGHS, EC 1.4.99.1), a rate-limiting enzyme for prostaglandin production. Two major isoforms of PGH synthase exist: PGHS-1 (Cox-1), a constitutive form; and PGHS-2 (Cox-2), an inducible form that is up-regulated in response to various inflammatory mediators (20). PGE₂ regulates the immune response, hematopoiesis, inflammation, tissue repair, and bone resorption. We have recently found that increases in PGE₂-releasing MØ are associated with the development of a Th1-to-Th2 shift of antigen-specific immune responses in a mycobacterial immunization model (19). The regulation of this shift may be, in turn, closely related to the regulation of PGE₂ release by MØ (4). The *in vivo* expression of PGE₂ release and the Th1 to Th2 shift, furthermore, may depend on the presence of an adequate number of PGE₂-MØ in the spleen (17). However, exact mechanisms of splenic PGE₂-MØ formation remain to be further elucidated.

The spleen is the secondary infectious site of mycobacteria and plays a systemic regulatory role in developing acquired immune responses against these pathogens. Unlike other tissue MØ isolated from peritoneum, bone marrow or blood, normal splenic MØ exhibit relatively low levels of PGE₂-releasing capacity (<1 ng of PGE₂/10⁶ MØ) (16,17). When normal splenic MØ are incubated with IFN γ and/or LPS, the primed MØ express PGHS-2 at relatively high levels but release PGE₂ at minimum levels (16). However, splenic PGE₂-releasing MØ (PGE₂-MØ, >10 ng of PGE₂/10⁶ MØ/ml) are increased by mycobacterial infections and other chronic disease conditions in which Th1-to-Th2 shifts are observed (5,10,13,22). Previous studies (15,17,19) indicate that heat-killed (HK) BCG and *Corynebacterium parvum* (*Propionibacterium acnes*), in a dose dependent manner, induce splenic PGE₂-MØ within 5 – 21 days following *i.p.* injection. Furthermore, the formation of splenic PGE₂-MØ is dependent on radiosensitive bone marrow cells but independent of circulating monocytes (15,17). Based on our previous study (15) using bone marrow cells adoptively transferred to ⁸⁹Sr-treated recipients, it is likely that, at the early stages (1 – 2 days) after HK-BCG immunization, precursors in the bone marrow migrate and localize to the spleen where mature forms of PGE₂-MØ are established. Alternatively, an inflammatory cytokine “milieu” may directly up-regulate PGE₂ biosynthesis by splenic MØ.

Adoptive transfer of donor cells including bone marrow cells is widely used in research on the function and metabolism of lymphoid and myeloid cells. In these types of immunological studies, it is often pertinent to quantify and visualize the adoptively transferred cells in immunological tissues of the recipient. It has been demonstrated that donor cells from green fluorescent protein (GFP) transgenic mice can be engrafted and visualized in recipient mouse tissue for over 22 weeks after transfusion (8,12). In this study, we evaluated whether splenic PGE₂-MØ are generated from adoptively transferred GFP⁺ donor bone marrow cells.

MATERIALS AND METHODS

Animals. Healthy 8- to 12-wk-old C57Bl/6 female mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Transgenic mice that express GFP (C57Bl/6-TgN [ACTbEGFP] 10sb) were provided by Dr. Kathryn Verbanac, East Carolina University, who obtained their breeding pairs from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained in barrier-filtered cages and fed Purina laboratory chow and tap water *ad libitum*. Experimental protocols employed in this study were approved by the IACUC of the Brody School of Medicine at East Carolina University and Florida Atlantic University.

Reagents. LPS from *Escherichia coli* (serotype 0111:B4, phenol extraction, L-2630) and mouse recombinant IFN γ were obtained from Sigma (I-4777, St. Louis, MO) and resuspended in pyrogen-free saline. A23187 (Sigma) was dissolved in dimethylsulfoxide (DMSO) at 1 mg/ml. Rabbit polyclonal anti-murine PGHS-1 and rabbit polyclonal anti-PGHS-2 were obtained from Cayman Chemicals (Ann Arbor, MI).

Preparations of HK-BCG. As described previously (19), the cultured bacteria of *M. bovis* Calmette-Guerin bacillus (BCG) Tokyo 172 strain were washed, autoclaved, and lyophilized. The HK-BCG powder was suspended in saline immediately before use. The suspensions were dispersed by brief (10s) sonication prior to injection. These HK-BCG preparations contained undetectable levels of endotoxin (<0.03 EU/ml), as determined by the *Limulus* amoebocyte lysate assay (Sigma, St. Louis, MO) (19).

Immunization of mice with HK-BCG and bone marrow transfusion. Groups of mice received 1 mg HK-BCG ip (5 mice/group) on day 0. Controls received 0.2 ml of saline. On Day 14, spleens and sera were harvested. To obtain bone marrow cells for transfusion, marrow cavities of femurs were flushed with ice-cold medium RPMI 1640; the expelled cell plugs were gently refluxed with a 20-gauge needle to form a single cell suspension. For bone marrow transfusion recipients, panels of 5 C57Bl/6 (wild-type [WT]) mice, 10 weeks of age, were immunized with 1mg HK-BCG ip. Immunized WT recipients received 2×10^7 bone marrow cells isolated from BCG-immunized GFP-donors. The transfusion of bone marrow cells was performed on days 1 and 2 (2×10^7 bone marrow cells/dose/day). In all experiments, bone marrow cells for transfer were prepared from a pool of 5 donors.

Splenic MØ preparations. Spleens from each group of mice were isolated and pooled. Excised spleens were minced with scissors and digested with 50 U/ml collagenase D (C-2139, Sigma) in RPMI 1640 plus 10% FBS at 37°C for 60 min followed by filtration through a 100- μ m mesh. After washing the cells with RPMI 1640 in the presence of DNase at 100 μ g/ml (DN-25, Sigma), cells were suspended in RPMI 1640 plus 10% FBS at 4×10^6 cells/ml. To enrich the MØ fraction (15,17), spleen cell suspensions were layered over a discontinuous Percoll gradient (35/60%, Sigma). Following centrifugation (800xg for 30 min, 22°C), cells in the layer between 35% and 60% Percoll were collected, washed and suspended in RPMI 1640 medium plus 10% FBS. These cells were plated at $3 - 5 \times 10^6$ cells per 60 mm culture dish (Falcon, Oxnard, CA) and incubated at 37°C in 5% CO₂ in air. After 2 hr incubation, the cells were washed with Ca²⁺ and Mg²⁺-free 0.15 M phosphate-buffered saline (PBS) for removal of non-adherent cells. Culture dishes were placed on ice for 30 minutes, following which the adherent cells were harvested by scraping with a cell scraper (Corning, Corning, NY) and washed twice with serum-free RPMI 1640. Viability was >90% (trypan blue exclusion). Adherent spleen cells

were >85% MØ, estimated by phagocytosis of IgG-opsonized sheep red cells and/or cytometrically following staining with anti-Mac-1 (17,18).

Western blotting. As described previously (16), freshly prepared or cultured splenic MØ were harvested and washed 3 times with cold saline. Washed cells were resuspended in lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, Sigma Protease Inhibitor Cocktail [1:500, P8340, Sigma], 1 % Nonidet P-40, and 1% sodium deoxycholate). Debris was eliminated by centrifugation (5 min, 10,000 x g). Protein concentration was measured using a commercial reagent based on bicinchoninic acid staining (Pierce, Rockford, IL) using BSA as a standard. Equal amounts of cellular protein were loaded onto SDS-polyacrylamide gels and separated by electrophoresis (200 V for 45 min). Proteins were then transferred to PVDF membranes (100 V for 1 h), and the membrane was blocked with 5% nonfat dry milk. The membranes were then incubated with primary antibody (anti-PGHS-1, 1:1000; anti-PGHS-2, 1:4000, Cayman, Ann Arbor, MI) overnight at 4°C. Following incubation with peroxidase conjugated donkey anti rabbit IgG (Jackson ImmunoResearch, West Grove, PA), proteins were detected by chemiluminescence (ECL, Amersham, Piscataway, NJ) per following the manufacturer's instructions.

Immunohistochemistry. Spleens were fixed with 4% paraformaldehyde overnight at 4°C, cryoprotected in 30% sucrose at 4°C for 24 hrs, and embedded in TBS freezing medium (Triangle Biomedical Sciences, Durham, NC) and stored at -70°C until use. Frozen spleens were cryosectioned at -20°C, thaw-mounted onto Plus-treated glass slides, heat-set at 30°C for 3 min, and then air dried overnight. For immunohistochemical analysis, 8-µm sections were stained with anti-PGHS-2 antibody at 1:250 (Cayman) followed by staining with tetramethyl rhodamine-conjugated donkey anti-rabbit IgG at 1:100 (Jackson ImmunoResearch). Fluorescence of each preparation was detected by a fluorescent microscope (Zeiss Photomicroscope III, Carl Zeiss, Inc. New York, NY).

PGE₂ release and detection. Splenic MØ (2 x 10⁶ cells/ml) prepared above were cultured in serum-free RPMI 1640 medium with 10⁻⁶ M calcium ionophore A23187 (Sigma) for 2 hrs. PGE₂ levels in the culture supernatants were measured by a competitive ELISA (Cayman).

Cytometric detection. The expression of surface antigens on spleen cell preparations was determined by indirect immunofluorescence in the presence of 5% heat-inactivated newborn calf serum (Sigma), pH 7.2 (18). Rat monoclonal antibodies used for the analyses were Mac-1 (β2 integrin), F4/80 (red pulp MØ), ER-TR9 (marginal zone MØ) and RB6-8C5 (neutrophil) (all from Research Diagnostics, Flanders, NJ). Phycoerythrin (PE)-conjugated donkey anti-rat IgG (Jackson ImmunoResearch) was used as a secondary antibody for indirect immunofluorescence.

To determine expression of cytosolic antigens, spleen cells prepared above were fixed with 4% paraformaldehyde, permeabilized with 1% saponin, and stained with rabbit antibodies specific for PGHS-1 and PGHS-2 at 2 µg/ml (Cayman) (18). Presence of the primary antibody was determined by PE-conjugated donkey anti-rabbit IgG at 1:1,000 (Jackson ImmunoResearch).

Fluorescence of 10⁴ stained cells, unless stated, was quantitated with a FACScan flow cytometer using the CellQuest program (Becton Dickinson, Mountain View, CA). All cells as defined by forward and sideward scatter pattern were gated; only debris was excluded from analysis. Cells stained with the second antibody alone were used as negative controls in all experiments.

Statistics. Data of PGE₂ release were analyzed by one way analysis of variance. For cell culture studies, tissues isolated from at least 4 mice were pooled unless indicated; these cells were cultured in at least triplicate in each group. *P* <0.05 is considered statistically significant.

RESULTS

PGE₂ release by splenic MØ isolated from HK-BCG-immunized C57Bl/6 (WT) mice.

Normal murine splenic MØ release PGE₂ at minimum levels compared to other tissue MØ including bone marrow MØ (15,16). Previous studies (15,17) suggest that HK-*C. parvum* and HK-BCG induce splenic PGE₂-MØ formation in a similar manner. To determine whether HK-BCG immunization increases PGE₂-MØ formation in the spleen, C57Bl/6 mice were immunized with 1 mg HK-BCG ip. Splenic MØ were isolated from mice days 0, 1, 2, 3 and 14 after immunization and stimulated *in vitro* with 1 µM calcium ionophore A23187 for 2 hrs. For comparison, normal splenic MØ were primed *in vitro* with LPS (10 µg/ml), IFNγ (10 ng/ml), LPS combined with IFNγ, or medium alone for 24 hr, followed by the elicitation of PGE₂ release with A23187 for 2 hrs.

As shown in Figure 1A, there was no increase in PGE₂ biosynthesis by splenic MØ on days 1, 2 and 3 after HK-BCG immunization. A 10-fold increase in PGE₂ release compared to the normal MØ PGE₂ level was observed when splenic MØ were isolated from C57Bl/6 mice 14 days after HK-BCG immunization. Priming of normal MØ with LPS and/or IFNγ *in vitro* did not significantly enhance PGE₂ biosynthesis (Figure 2). These results extend and confirm our previous reports (16).

Immunization with HK-BCG enhances PGHS-2 expression by splenic MØ. To determine whether increases in PGE₂ release by splenic MØ are associated with increased expression of PGHS-1 and PGHS-2, these proteins were detected by Western blotting. As a comparison, PGHS-1 and PGHS-2 in normal MØ that were primed with LPS and/or IFNγ *in vitro* were also determined.

MØ samples isolated from days 0 (unimmunized), 1, 2 and 14 after immunization expressed PGHS-1 at comparable levels (Figure 1B). Levels of PGHS-2 in normal MØ were undetectable; however, PGHS-2 levels were increased on days 1 and 14, but on day 2 were significantly lower compared to those on days 1 and 14 (Figure 1B), suggesting bi-phasic PGHS-2 induction by HK-BCG immunization. Increases in PGHS-2 expression were also observed for LPS-primed normal MØ. Priming with a mixture of LPS and IFNγ further enhanced PGHS-2 levels (Figure 2). Furthermore, immunohistological analyses also showed that remarkable numbers of PGHS-2⁺ spleen cells were detected on days 1 and 14 after HK-BCG immunization (Figure 1C). However, there was no PGHS-2⁺ cells detected on days 0 (unimmunized) and 2 (data not shown). Taken together, our results indicate that MØ isolated 14 days after HK-BCG immunization showed relatively high PGE₂ release and PGHS-2 expression. In sharp contrast, MØ that were isolated from mice 1 day after HK-BCG immunization also express PGHS-2 despite only a minimal increase in PGE₂ biosynthesis (Figure 1). Such dissociation between PGHS-2 expression and PGE₂ biosynthesis was also observed for normal MØ that were primed with LPS or LPS mixed with IFNγ *in vitro* (Figure 2).

GFP-positive PGE₂-MØ in Spleen. Previous studies (15,17) indicate that HK-*C. parvum* induces splenic PGE₂-MØ formation in a radiosensitive bone marrow cell-dependent manner. Specifically, there was no splenic PGE₂-MØ formation when mice were depleted of bone marrow cells by ⁸⁹Sr before HK-*C. parvum* immunization. Furthermore, transfusion of bone marrow cells isolated from donors that were immunized with HK-*C. parvum* resulted in recovery of PGE₂-MØ formation in mice treated with ⁸⁹Sr. Effective recovery requires bone marrow transfusions on days 1 and 2 after HK-*C. parvum* immunization in both donors and recipients. To assess whether HK-BCG-induced splenic PGE₂-MØ are derived from transfused donor cells, we established a bone marrow chimera using HK-BCG-immunized WT (C57Bl/6) mice as recipients and HK-BCG-immunized GFP-transgenic mice as donors. Both recipients and donors were immunized with 1 mg HK-BCG ip on day 0. Bone marrow cells were isolated and transfused immediately on days 1 and 2 (2 x 10⁷ cells/day).

Bone marrow cells, spleen cells and peritoneal cells were prepared from HK-BCG-immunized chimeras and GFP expression was determined cytometrically. Figure 3 shows that small, but significant numbers of GFP⁺ cells were detected in the tissues isolated from the chimera (Table 1). The results in Figure 3 indicate that the

chimera in animals immunized with HK-BCG resulted in higher engraftment of donor cells than for the non-immunized chimera. Our results suggest that immunization of mice with HK-BCG is essential to induce migration, localization and differentiation of donor cells.

Two-color cytometric analyses showed that 0.29% spleen cells expressed both GFP and PGHS-2, suggesting that PGE₂-MØ originated from donors, whereas 10.67% spleen cells appeared to be recipient PGE₂-MØ (Table 1, Figure 4). Selected MØ populations characterized by monoclonal antibodies against Mac-1 (β2 integrin), F4/80 (red pulp MØ) and ER-TR9 (marginal zone MØ) are generated from transfused bone marrow cells (Table 1). Figure 5 illustrates PGHS-2⁺ spleen cells expressing GFP characterized immunohistologically.

DISCUSSION

The distinctive features of the formation of PGE₂-releasing splenic MØ described in this paper may be summarized as follows: (i) normal splenic MØ from C57Bl/6 mice express relatively low levels of PGE₂ (<0.6 ng of PGE₂/10⁶ MØ/ml) whereas splenic MØ isolated from mice 14 days after immunization with HK-BCG show a 10-fold higher PGE₂ release (>6 ng/ml) (Figure 1); (ii) the priming of normal splenic MØ with LPS or LPS plus IFNγ *in vitro* enhances PGE₂ synthesis to about 1 ng/ml and PGHS-2 (Cox-2) protein expression (Figure 2); (iii) splenic MØ isolated on day 1 after HK-BCG immunization, splenic MØ express PGHS-2 at high levels without an increase in PGE₂ synthesis (Figure 1); and (iv) bone marrow-derived MØ may contribute to the increased PGE₂ production and PGHS-2 expression observed for splenic MØ 14 days after BCG immunization (Figures 4 and 5).

Populations or compartments of mononuclear phagocytes (MØ) show considerable diversity of cellular function including PGE₂ biosynthesis. Treatment with bacterial components *in vitro* and *in vivo* can enhance PGE₂ biosynthesis indicating that immunomodulation is also an important determinant of MØ diversity. Employing mice depleted of bone marrow cells and circulating monocytes by the bone seeking isotope ⁸⁹Sr, we previously demonstrated that PGE₂-MØ isolated from the spleen and peritoneal cavity are ontogenically distinct (15,17). The high PGE₂-releasing capacity of resident peritoneal MØ is independent of radiosensitive bone marrow cells. In contrast, splenic PGE₂-MØ formation is dependent on radiosensitive bone marrow. To further explore the role of bone marrow-derived cells, we have used adoptive transfer of functional GFP-positive bone marrow cells into recipients. Based on previous results (15), the functional bone marrow cells were obtained from GFP-transgenic donors 1 and 2 days after HK-BCG. Our results clearly show that transfusion of WT recipients with GFP⁺ bone marrow cells establishes a GFP-chimera in bone marrow, spleen and peritoneal cells and indicates that HK-BCG immunization induces and establishes not only splenic PGE₂-MØ formation but also other bone marrow-derived cell populations. The provocative findings in this study, however, are that splenic PGE₂-MØ may be derived from GFP⁺ bone marrow cells that are transfused on days 1 and 2 after HK-BCG immunization and that bone marrow does not solely supply an appropriate cytokine milieu. These results without the use of ⁸⁹Sr to deplete the recipient bone marrow further confirm our earlier conclusion that PGE₂-MØ in the spleen, following exposure to mycobacterial products, originate from bone marrow precursors.

Various inflammatory mediators including IFNγ, IL-1, and TNFα as well as the bacterial endotoxin LPS induce PGHS-2 in many MØ and non-MØ populations. IFNγ in synergy with LPS is known to induce PGHS-2 in bone marrow MØ and peritoneal MØ (2), through the activation of NF-κB (11). In the present study, we found that normal splenic MØ in C57Bl/6 mice express PGHS-1 but not PGHS-2, and release less than 0.6 ng/ml of PGE₂. On the other hand, splenic MØ that are isolated from mice 14 days after HK-BCG immunization express both PGHS-1 and PGHS-2, and release over 6 ng of PGE₂/10⁶ of MØ. NS-398 and nimesulide, both PGHS-2 inhibitors, inhibit PGE₂ release by splenic PGE₂-MØ (16). It is reasonable to speculate, therefore, that PGHS-2 in splenic PGE₂-MØ is necessary for increased PGE₂ biosynthesis.

However, PGHS-2 is not sufficient for increased splenic PGE₂ production because over expression of PGHS-2 protein is induced both in 1 day splenic MØ following HK-BCG immunization and in normal splenic MØ treated *in vitro* with LPS and IFN γ despite little or no increase in PGE₂ biosynthesis. Previous studies by Kuroda and Yamashita (7) and ours (16) demonstrated such dissociation between PGHS-2 expression and PGE₂ biosynthesis in the *in vitro* activation of splenic MØ isolated from Balb/c, C57Bl/6 and IL-10^{-/-} mice. Kuroda and Yamashita (7) also suggested that certain tumor cells express high levels of PGHS-2 with little PGE₂ biosynthesis. Since the PGHS-1 level is relatively unchanged in our studies, PGE₂ synthase (PGES) (9,23) and/or phospholipase A₂ (6,7) must be involved as key rate-limiting determinants required for splenic PGE₂-MØ formation and would be found in insufficient amounts in the day 1 splenic MØ. One cannot rule out that PGE₂-MØ formation would include a contribution from PGHS-3, a PGHS-1 variant that has been identified more recently (3).

PGE₂ is a key mediator regulating inflammatory responses (1,5,14,21). Under chronic inflammatory conditions, increases in splenic PGE₂-MØ potentially induce the Th1-to-Th2 shifts of the immune response that is a major pathogenic event in mycobacterial infections and other chronic inflammatory diseases. The mechanisms regulating PGE₂ synthesis by MØ at local sites of inflammation appear to be complex. Additional bone marrow-dependent mechanisms appear to participate in formation of splenic PGE₂-MØ isolated from mice 7 – 14 days after BCG immunization. These results with a GFP bone marrow chimera are consistent with the previous hypothesis that splenic PGE₂-MØ are derived from radiosensitive bone marrow cells (15,17).

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Footnote

Abbreviations used in this paper: PGHS, prostaglandin G/H synthase; WT, wild-type; MØ, macrophages; CMI, cell-mediated immunity; BCG, Bacillus Calmette-Guérin; PGE₂, prostaglandin E₂; HK, heat killed

Table 1. Percent of spleen cells expressing selected cellular antigens.

	WT recipient	GFP-transgenic donor		GFP-chimera	
	GFP-negative	GFP-negative	GFP-positive	GFP-negative	GFP-positive
Total cell	100%	27.96%	72.04 %	98.73%	1.27%
PGHS-1	39.48	0.56	13.14	37.53	0.50
PGHS-2	7.89	0.05	5.18	10.67	0.29
Mac-1 ($\beta 2$ integrin)	32.83	10.03	8.11	18.74	0.54
F4/80 (red pulp MØ)	2.63	0.28	3.76	9.89	0.52
ER-TR9 (marginal zone MØ)	1.67	0.37	2.49	1.02	0.26
RB6-8C5 (neutrophil)	16.18	0.98	25.02	19.65	0.77

WT (C57Bl/6) recipients and GFP-transgenic donors were immunized with HK-BCG. Bone marrow cells (2×10^7 cells/dose) from donors were transfused into the recipients on days 1 and 2 after HK-BCG immunization. On day 14, recipients were sacrificed and spleen cells were isolated. Spleen cells were also isolated from separated groups of WT recipients and GFP-transgenic donors 14 days after HK-BCG immunization. Following staining with PE-labeled antibodies against selected cellular antigens, their fluorescence activities were determined cytometrically as shown in Figure 4.

FIGURE LEGENDS

Figure 1. HK-BCG induces splenic PGE₂-MØ formation in C57Bl/6 mice. Groups of C57Bl/6 mice received 1 mg HK-BCG ip (4 mice/group) on day 0. At indicated intervals, spleens were harvested and splenic MØ were isolated and pooled in each group. (A) To determine PGE₂ biosynthesis, MØ suspended in serum-free RPMI 1640 were incubated with 1 μ M A23187 for 2 hrs. PGE₂ levels in the supernatants were measured by ELISA. Mean \pm SD, n=3. * and **, $p < 0.05$ and $p < 0.001$, respectively, compared to day 0 control group. Each result represents from three experiments. (B) Proteins were extracted and western blotting was performed staining with anti-PGHS-1 or anti-PGHS-2; each lane was loaded with 5 μ g total protein. Results are representative of three separate experiments. (C) To detect PGHS-2⁺ cells immunohistologically, spleen were fixed, frozen and sectioned as described in the Materials and Methods. The sections were stained with anti-PGHS-2 primary antibody followed by tetramethyl rhodamine-conjugated second antibody. Results (10x magnification) are representative of three separate experiments.

Figure 2. PGHS-2 levels and PGE₂ biosynthesis in splenic MØ that were primed with IFN γ and/or LPS *in vitro*. Splenic MØ (5×10^6 /ml) isolated from C57Bl/6 mice were primed with LPS (10 μ g/ml), IFN γ (10 ng/ml), LPS mixed with IFN γ , or medium alone for 24 hr. Method was as described for Fig. 2. Results are representative of three separate experiments. To determine PGE₂ biosynthesis, MØ were incubated with 1 μ M A23187 for 2 hrs and PGE₂ levels in the supernatants were measured by ELISA. Mean \pm SD, n=3.

Figure 3. Chimeras for bone marrow, spleen and peritoneal cells in C57Bl/6 recipients that received bone marrow cells from GFP-transgenic donors. WT recipients and GFP-transgenic donors were immunized with HK-BCG. As control groups, recipients and donors were immunized with saline. Bone marrow cells (2×10^6 cells/dose) from donors were transfused into the recipients on Days 1 and 2 after immunization. On Day 14, recipients were sacrificed and bone marrow cells, spleen cells and peritoneal lavage cells were isolated. GFP levels were determined cytometrically. As controls, bone marrow cells, spleen cells and cells in peritoneal

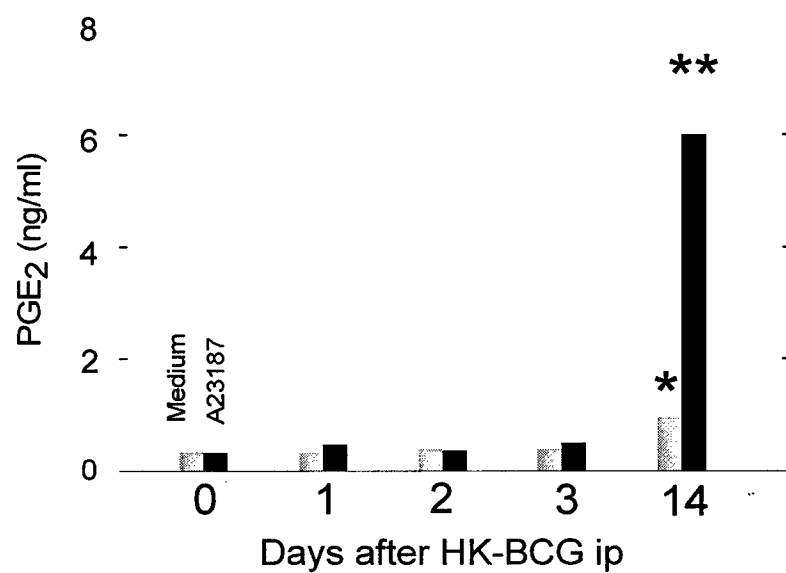
- lavage were prepared from WT recipients and GFP-transgenic mice 14 days after HK-BCG immunization. Cellular fluorescence was determined by cell sorter with emission at 480 nm and excitation at 530 nm.

Figure 4. Cytometric detection of spleen cells expressing both GFP and PGHS-2 in the HK-BCG-immunized GFP-chimera. GFP-chimera mice following immunization with HK-BCG were prepared as indicated in Figure 3. Spleen cells were isolated from (A) GFP-chimera, (B) WT recipients and (C) GFP-donor controls. Intracellular PGHS-1 and PGHS-2 were stained with anti-PGHS-1, anti-PGHS-2 and normal rabbit IgG (as negative control) followed by PE-conjugated donkey anti-rabbit IgG. Fluorescence of 10^5 stained cells was quantitated cytometrically. PE-positive cells and GFP-positive cells were counted by two-color analysis.

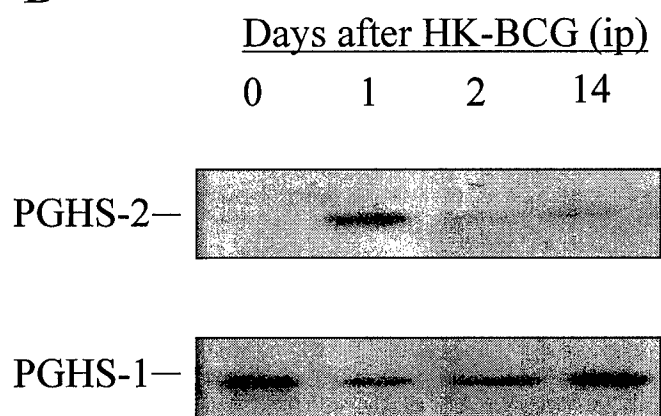
Figure 5. Immunofluorescent detection of spleen cells expressing both GFP and PGHS-2 in the HK-BCG-immunized bone marrow chimera. A green fluorescent protein (GFP)-bone marrow chimera was established in C57Bl/6 recipients that received bone marrow cells intravenously from GFP-donors. Both C57Bl/6 recipients and GFP-transgenic donors were immunized with 1 mg HK-BCG ip on Day 0. On Days 1 and 2, bone marrow cells were isolated from GFP-donors and transfused into C57Bl/6 recipients at 2×10^7 cells/dose/day. On Day 14, spleens were harvested from C57Bl/6 recipients. To detect PGHS-2⁺ cells immunohistologically, spleen were fixed, frozen and sectioned as described in the Materials and Methods. The sections were stained with anti-PGHS-2 primary antibody followed by tetramethyl rhodamine (red)-conjugated second antibody. In the two sections with 100x magnification, GFP⁺ spleen cells (green) were co-localized with PGHS-2⁺ cells (red). Without HK-BCG immunization in the donors and recipients, there was no PGHS-2⁺ cells or GFP⁺ cells in the spleen (data not shown).

Figure 1.

A



B



C

Day 1



Day 14



Figure 2

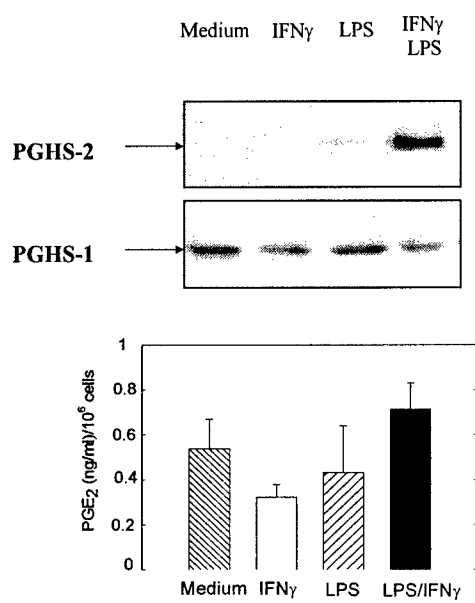


Figure 3.

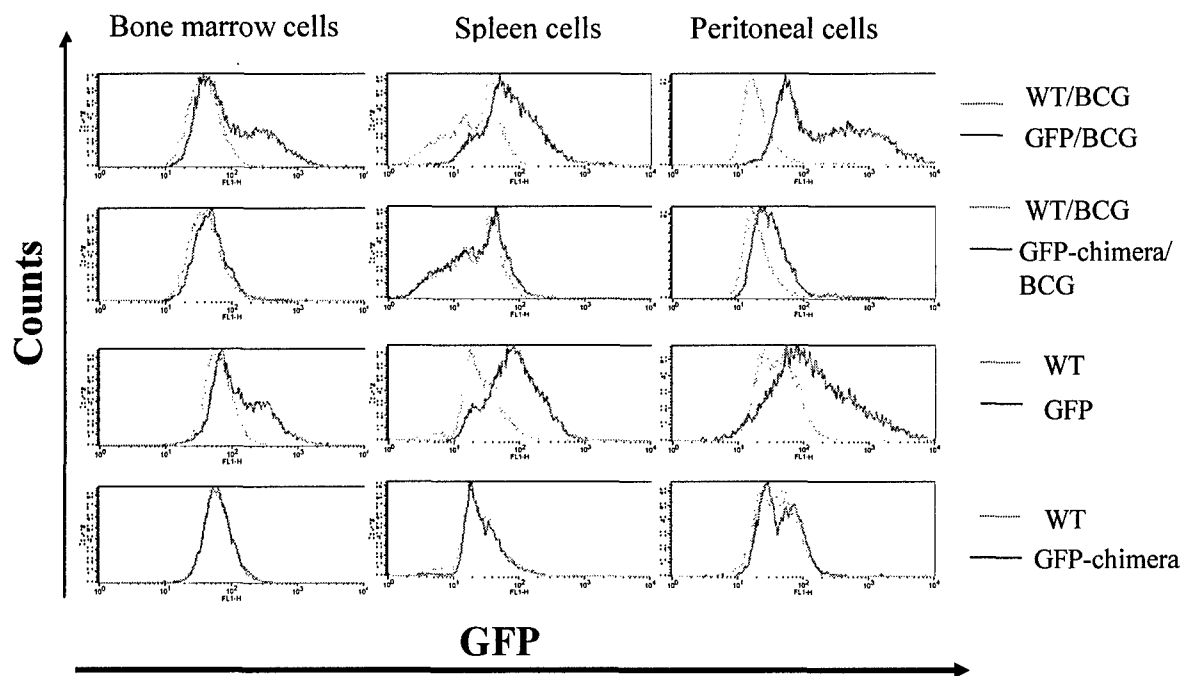
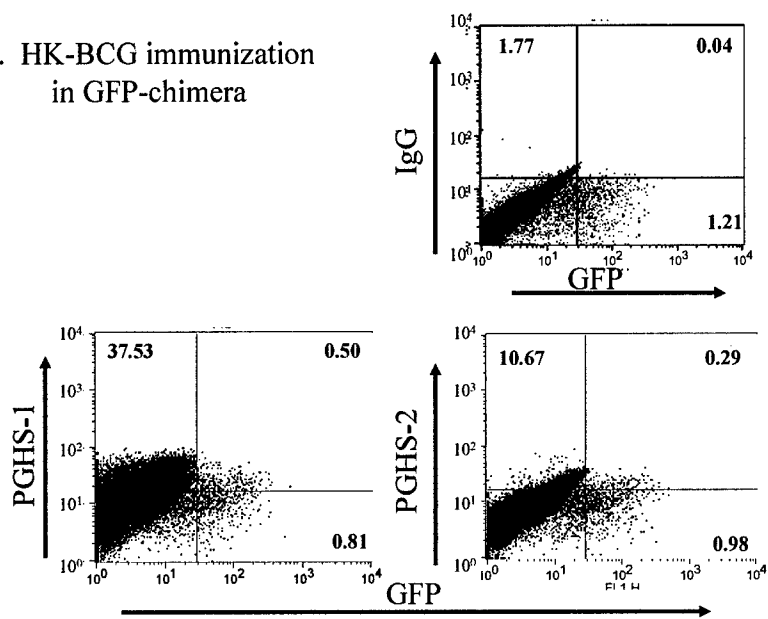
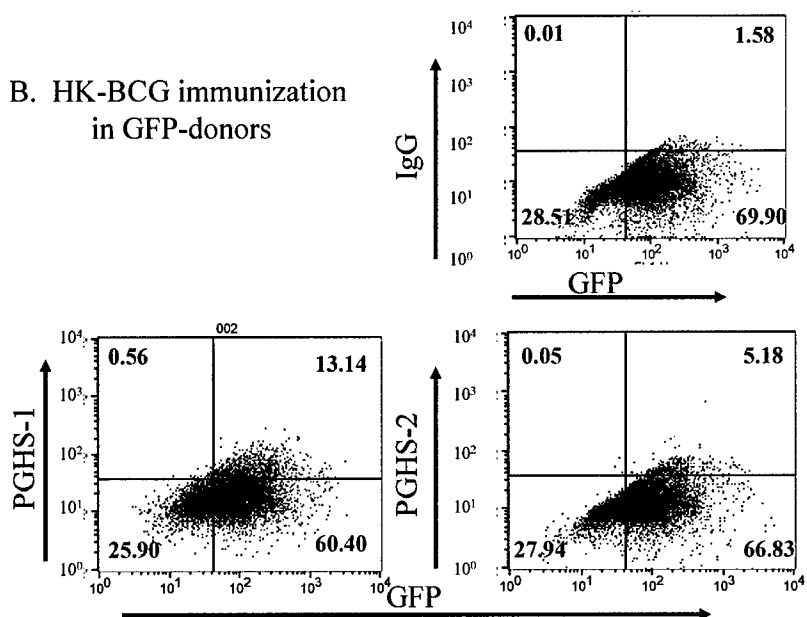


Figure 4.

**A. HK-BCG immunization
in GFP-chimera**



**B. HK-BCG immunization
in GFP-donors**



C. HK-BCG immunization
in WT recipients

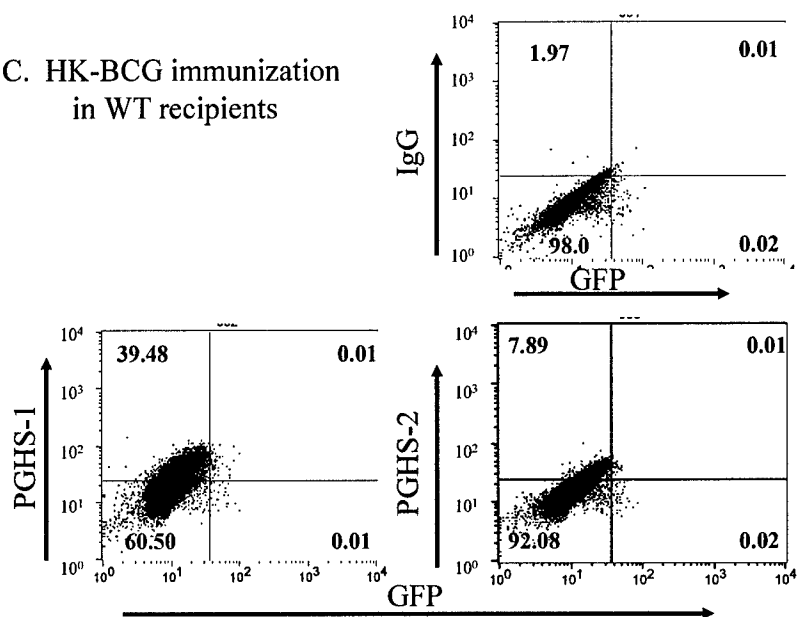
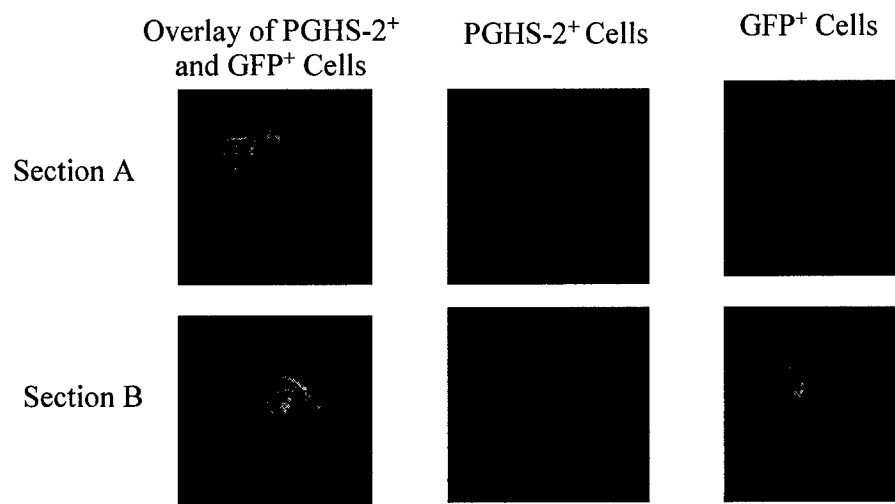


Figure 5



Appendix III

Mitogen-activated protein kinase activation is associated with TNF- α , IL-10, and IL-1 β production by RAW264.7 cells phagocytosing chitin particles

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ABSTRACT

When mouse macrophages (MØ) phagocytose *N*-acetyl- β -D-glucosamine polymer (chitin) particles, they produce cytokines including TNF- α , IL-10, and IL-1 β . We determined whether 1 – 10 μ m chitin particles activate mitogen-activated protein kinase (MAPK) families that regulate the production of TNF- α , IL-10, and IL-1 β . We used RAW264.7 MØ-like cells, in which p38 MAPK (p38), c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (Erk) 1/2 are activated by bacterial endotoxin (LPS) and CpG-containing oligonucleotides (CpG-ODN). We found that 1 – 10 μ m chitin particles also activated p38, JNK, and Erk 1/2 with production of large amounts of TNF- α and IL-1 β and a lesser amount of IL-10. Soluble chitin, larger sizes of chitin (>50 μ m), chitosan (de-acetylated chitin) particles (1 – 10 μ m), or latex beads (1.1 μ m) failed to induce either MAPK activation or cytokine production. Inhibitory studies with SB203580, PD98059, and SP600125 showed that both p38 and JNK pathways are involved in chitin-induced IL-1 β and TNF- α production whereas all three pathways are essential for IL-10 production. The contribution of each MAPK family for IL-1 β and IL-10 production was distinct among the agonists. Taken together, MAPK activation contributes to MØ cytokine production when MØ phagocytose micro-particles through recognition of *N*-acetyl- β -D-glucosamine residues.

INTRODUCTION

In the innate immune response, phagocytosis by local macrophages (MØ) is the first line of defense against invading microorganisms. The phagocytosis of unopsonized microorganisms results in inflammatory cytokine production leading to development of innate immunity and microbicidal MØ. MØ also phagocytose non-microbial particles including cell debris, environmental particulates and materials derived from prostheses. MØ recognize particles through plasma membrane receptors, such as C-type lectin receptors (CLR) (25, 31), integrins (44), scavenger receptors (15), and toll-like receptors (TLR) (1, 22), which results in the particle internalization. However, soluble bacterial LPS and DNA, for example, induce MØ inflammatory activation and establish innate immune defenses through TLR4 and TLR9, respectively (1, 22, 47). Transmembrane signaling without particle internalization is well demonstrated in MØ recognizing pathogens or their components through these receptors (10, 16). In contrast, although phagocytosis of microbial particles through CLR plays an important role in innate immune responses (25, 31), the mechanisms of MØ cytokine production remain to be elucidated.

Previously, we found that normal mouse splenic MØ phagocytose 1 – 10 μ m chitin (*N*-acetyl- β -D-glucosamine polymer) particles through CLR, MØ with production of TNF- α , IL-1 β , IL-10, IL-12, and IL-18 (35-39). Chitin particle-induced cytokine production is inhibited by pre-treatment of MØ with soluble mannan, a CLR ligand, or cytochalasin D, an actin polymerization inhibitor. However, the receptor ligation by small soluble chitin or large chitin particles (> 50 μ m) fails to induce cytokine production. Furthermore, phagocytosis of particles of chitosan (de-acetylated chitin) or latex fails to induce cytokine production. Our studies indicate that both CLR ligation and internalization of chitin particles are required for cytokine production, although exact mechanisms of cellular signaling are unknown.

LPS, unmethylated CpG-containing oligonucleotide (CpG-ODN), live *Mycobacterium avium*, and live *Chlamydia pneumoniae* activate mitogen-activated protein kinases (MAPK) in various MØ populations including RAW264.7 MØ-like cells (2, 17, 20, 32, 34, 47). MAPK family consists of p38 MAPK (p38), extracellular signal-regulated kinase (Erk) 1/2, and c-Jun N-terminal kinase (JNK). Activation of one or more of these families appears to regulate cytokine production including IL-1 β , IL-10 and TNF- α (4, 32, 34, 47). In the present study, we investigated whether p38, Erk 1/2, and JNK phosphorylation are activated when RAW264.7 cells phagocytose 1 – 10 μ m chitin particles or phagocytosable particles including chitosan and polystyrene latex beads. We also investigated whether soluble chitin or >50 μ m chitin particles, ligands to CLR, induces MAPK activation. Finally, we determined whether the activation of MAPK is obligatory for chitin particle-induced cytokine production. The roles of MAPK for cytokine production were compared among chitin particles, LPS, and CpG-ODN. Our results indicate that chitin particles induce MAPK activation

in RAW264.7 cells at levels comparable to those of LPS or CpG-ODN. However, the contribution of each MAPK family for cytokine production is distinct among the agonists.

MATERIALS AND METHODS

Reagents and antibodies. Chitin powder was purchased from Sigma (St. Louis, MO) and phagocytosable (1 – 10 μ m) and non-phagocytosable (> 50 μ m) chitin particles were prepared as described previously (36). Soluble chitin oligosaccharide was kindly provided by Kyowa Technos (Chiba, Japan). Chitin particles, 1 – 10 μ m, were de-acetylated with 24% sodium hydroxide at 55°C for 16 hrs, washed three times with saline and used as 1 – 10 μ m chitosan particles. Latex beads (1.1 μ m, polystyrene) and LPS (*Escherichia coli* serotype 0111:B4, phenol extraction) were purchased from Sigma (St. Louis, MO). Both endotoxin-free CpG-ODN (5' TCC ATG ACG TTC CTG ACG TT 3'; unmethylated) and GpC-ODN (5' TCC ATG AGC TTC CTG AGC TT 3'; unmethylated) were purchased from TriLink (Sorrento Mesa, CA). Each of the stimulating reagents was suspended or dissolved in endotoxin-free saline as a 10 mg/ml stock solution. Selective p38 inhibitor SB203580 was purchased from Sigma. Selective Erk 1/2 pathway inhibitor PD98059 and JNK/SAPK inhibitor SP600125 were purchased from Calbiochem (La Jolla, CA). The inhibitors were dissolved in DMSO to prepare 10 mM stock solutions. Stock solutions of all inhibitors were stored at -20 °C until use. Rabbit polyclonal anti-p38, anti-phospho-p38 (anti-p-p38), anti-Erk 1/2, anti-phospho-Erk 1/2 (anti-p-Erk 1/2), anti-JNK, and anti-phospho-JNK (anti-p-JNK) antibodies were purchased from Cell Signaling Technology (Beverly, MA).

Cell culture and MAPK activation. Murine MØ-like RAW264.7 cells (American Type Culture Collection, Manassas, VA) were grown in RPMI1640 (Life Technologies, Gaithersburg, MD) containing 5% heat-inactivated fetal bovine serum (FBS), penicillin G, streptomycin, and amphotericin B at 5% CO₂ and 37 °C in a 100% humidified incubator. Cells were passaged every 2 - 3 days to maintain logarithmic growth until they were used. For MAPK activation studies, cells (1×10^6 cells/ml) were incubated in serum-free RPMI1640 for 2 hr before stimulation. Cells were then stimulated with 1,000 μ g/ml 1 – 10 μ m chitin particles, 1,000 μ g/ml >50 μ m chitin particles, 1,000 μ g/ml soluble chitin oligosaccharide, 1,000 μ g/ml 1 – 10 μ m chitosan particles, 1,000 μ g/ml 1.1 μ m latex beads, 1 μ g/ml LPS, 5 μ g/ml CpG-ODN, or 5 μ g/ml GpC-ODN. Cells were incubated at 37 °C for 0, 10, 20, 30, and 40 min.

Protein extraction and western blotting. Cells were lysed with SDS-lysis buffer (50 mM Tris-HCl, pH 7.5, 10 mM NaF, 1 mM Na₃VO₄, 5 mM EDTA, and 1% SDS) by heating at 95 °C for 5 min and chilled on ice. Debris in the lysate was eliminated by centrifugation (15,000 g, 3 min). Protein concentration in the supernatant was measured by a bicinchoninic acid reagent (Pierce, Rockford, IL) using bovine serum albumin as standard. Equal amounts of cellular protein were loaded onto SDS-11% polyacrylamide gel and separated by electrophoresis. Separated proteins were then electroblotted onto PVDF membrane. After blocking with non-fat dry milk, total MAPK and dual phosphorylated MAPK were stained with antibodies against total MAPK (anti-p38, anti-Erk 1/2, or anti-JNK) and dual phosphorylated MAPK (anti-p-p38, anti-p-Erk1/2, or anti-p-JNK), respectively. Following incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch, West Grove, PA), MAPK and dual phosphorylated MAPK were detected by chemiluminescence (ECL Western Blotting Detection Reagents, Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

Cytokine production. RAW264.7 cells at 5×10^5 cells/ml were stimulated with 100 μ g/ml 1 – 10 μ m chitin particles, 0.1 μ g/ml LPS, 5 μ g/ml CpG-ODN, or saline in 96-well plates at 37°C and 5% CO₂. After 3, 6, or 24 hr, culture supernatants were harvested and stored at -80°C until used for ELISA. To assess the effects of SB203580, PD98059 and SP600125 on cytokine production, cells were pretreated with each MAPK inhibitor or DMSO at 37°C for 30 min prior to stimulation with chitin particles.

ELISA. Levels of TNF- α , IL-10, and IL-1 β in the culture supernatant were measured by the two-site ELISA specific for the respective cytokines (BD PharMingen, San Diego, CA).

Endotoxin removal. Endotoxin was removed from soluble materials for culture by filtration and sterilization through a 0.22- μ m-pore-size Zetapore membrane (AMF-Cuno; Cuno, Meriden, CT). The final preparations were monitored for endotoxin by the *Limulus* amoebocyte assay (Sigma).

Statistics. Differences between mean values were analyzed by Student's *t* test. *P* value less than 0.05 is considered statistically significant.

RESULTS

Chitin particle-induced TNF- α , IL-10, and IL-1 β production in RAW264.7 cells. We determined whether RAW264.7 cells produce TNF- α , IL-10, and IL-1 β in response to 1 – 10 μ m chitin particles, LPS, or CpG-ODN. As shown in Fig. 1, 1 – 10 μ m chitin particles stimulated production of relatively large amounts of TNF- α and IL-1 β within 3 hr that further increased at 6 and 24 hr. Chitin particles, however, induced a minimum amount of IL-10 production within 24 hr. In contrast, LPS induced large amounts of TNF- α , IL-10, and IL-1 β in a time-dependent manner. CpG-ODN-stimulated cells produced TNF- α and IL-10 but a minimum level of IL-1 β . GpC-ODN, an inactive oligonucleotide control, did not induce production of these cytokines within 24 hr.

Soluble chitin oligosaccharide or non-phagocytosable chitin particles (> 50 μ m) induced only minimum levels of TNF- α production (Fig. 1). However, these agonists failed to induce production of either IL-1 β or IL-10. Furthermore, 1 – 10 μ m chitosan particles and 1.1 μ m latex beads induced minimal or no production of the three cytokines (Fig. 1). Our results indicate that chitin-induced M ϕ responses are dependent specifically on the recognition of chitin and not de-acetylated chitin or polystyrene components.

Chitin particles induced MAPK phosphorylation in RAW264.7 cells. We determined whether MAPK families are activated by chitin particles and other agonists. As reported previously (11, 13, 26, 36), we confirmed that LPS or CpG-ODN induced p38, Erk 1/2, and JNK phosphorylation within 20 min (Fig. 2). GpC-ODN did not induce MAPK phosphorylation for 40 min after stimulation. Chitin particles at 1 – 10 μ m sizes also induced p38, Erk 1/2, and JNK phosphorylation. The peak of phosphorylation was 30 min for p38 and 30 and 40 min for both Erk 1/2 and JNK (Fig. 2). In sharp contrast, soluble chitin oligosaccharide, > 50 μ m chitin particles, 1 – 10 μ m chitosan particles, and latex beads did not activate MAPK.

Effects of MAPK inhibitors on TNF- α production. To investigate the roles of each MAPK pathway for cytokine production, cells were pre-treated with SB203580 (p38 inhibitor), PD98059 (Erk 1/2 pathway inhibitor), or SP600125 (JNK inhibitor) at 37 °C for 30 min before agonist stimulation. As shown in Fig. 3A – C, chitin particle-induced TNF- α in 3 hr culture was inhibited with SB203580 or SP600125. Both SB203580 and SP600125 also inhibited LPS- or CpG-ODN- induced TNF- α production at 3 hr (Fig. 3A – C). However, PD98059 failed to inhibit TNF- α production at 3 hr (Fig. 3A – C).

Interestingly, inhibitory effects on TNF- α production were time-dependent. As shown in Fig. 3D, the inhibition by SB203580 was not shown in the chitin particle-induced TNF- α production in 6- or 24- hr cultures. The inhibitory effect of SP600125 on LPS-induced TNF- α production was decreased in a time-dependent manner. Unlike in 3- and 6- hr cultures, LPS-induced TNF- α production in 24-hr culture was up-regulated by the pretreatment with PD98059 (Fig. 3E). The inhibitory effects of SB203580 and SP600125 on CpG-ODN-induced production were decreased in a time-dependent manner. PD98059 showed significant reduction of CpG-ODN-induced TNF- α production in 24 hr culture (Fig. 3F).

Effects of MAPK inhibitors on IL-10 production. Since IL-10 levels at 3 and 6 hrs were very low, the results of 24 hr treatment are presented. As shown in Fig. 4, each inhibitor reduced chitin particle- or LPS- induced IL-10 production. However, CpG-ODN-induced IL-10 production was inhibited only by SB203580 and SP600125. In contrast, 10 μ M PD98059 enhanced CpG-ODN-induced IL-10 production.

Effects of MAPK inhibitors on IL-1 β production. As shown in Fig. 5, chitin particle-induced IL-1 β production in the 24-hr cultures was slightly but significantly inhibited by SB203580 or SP600125. PD98059 reduced chitin particle-induced IL-1 β production, but the effect was insignificant. In sharp contrast, all three inhibitors enhanced, rather than inhibited, LPS-induced IL-1 β production. CpG-ODN did not induce IL-1 β production (Fig. 1 and 5) and this was not altered by any of the three MAPK inhibitors (Fig. 5).

DISCUSSION

N-acetyl- β -D-glucosamine sugar residues are recognized by mannose-type CLR including mannose receptors, endo-180 and langerin C-type receptors (25, 31). These receptors, however, have significantly lower binding affinities to de-acetylated glucosamine sugar residues (31). Dectin-1/ β -glucan CLR, on the other hand, recognizes β -1, 3- and β -1, 6-linked glucan residues but not mannose or *N*-acetyl- β -D-glucosamine residues (3, 25, 42). We originally found (35, 36), and other groups (18, 41) confirmed, that phagocytosable *N*-acetyl- β -D-glucosamine polymer (chitin), a seemingly inert molecule, induces the activation of M ϕ leading to an innate immune response. Chitin-induced innate immunity not only develops microbicidal M ϕ formation but also, when mixed with water-soluble antigens, induces acquired Th1 lymphocyte responses (37-39) and reduces Th2 lymphocyte responses (38, 41). Chitin particles bind to CLR on M ϕ plasma membranes and the receptors and particle complexes are internalized. The present study has demonstrated that the phagocytic process activates MAPK, which is obligatory for cytokine production. However, receptor ligation by soluble chitin or non-phagocytosable chitin particles fails to induce the activation of MAPK or the production of cytokines. We further demonstrated that phagocytosis of de-acetylated glucosamine particles does not induce either MAPK activation or production of the selected cytokines.

We have confirmed previous observations (17, 20, 34, 47) that LPS and CpG-ODN induce RAW264.7 cells to activate p38, Erk 1/2, and JNK and have shown that the patterns induced by chitin particles are similar (Fig. 2). However, although RAW264.7 cells have been widely used in various immunological studies as a model of murine M ϕ , their reported phenotypes are varied. These include the expression of mannose receptors and the production of IL-12p70, IL-10, IL-1 β , and TNF- α (4, 23, 26, 34, 40, 48). Our cytometric studies with anti-mannose receptor antibody (a gift from Dr. Philip Stahl, Washington University, St. Louis, MO) have shown that 15-20 % of our RAW264.7 cells expressed mannose receptors, compared to 60-70 % for normal splenic M ϕ (data not shown). Furthermore, in our hands, RAW264.7 cells produced undetectable amounts (<10 pg/ml/10⁶ cells) of IL-12p40 or IL-12p70 in responding to chitin particles, LPS or CpG-ODN (data not shown). Previous studies showed that all agonists induce IL-12p70 in splenic M ϕ isolated from normal mice (36).

Our studies have clearly demonstrated that chitin particles stimulate TNF- α production by RAW264.7 cells at levels and with kinetics comparable to LPS or CpG-ODN. However, IL-10 and IL-1 β are produced in an agonist-dependent manner. Chitin induces only a small amount of IL-10 at 24 hrs. IL-1 β production is not induced by CpG-ODN. Since comparable levels of MAPK activation are induced by chitin particles, LPS and CpG-ODN, we determined the contribution of each MAPK family to cytokine production employing SB203580 (p38 inhibitor), PD98059 (ERK1/2 pathway inhibitor) and SP600125 (JNK/SAPK inhibitor). Fig. 6 summarizes our results. When RAW264.7 cells are stimulated with chitin particles, p38 regulates IL-1 β , IL-10 and TNF- α production; Erk 1/2 regulates IL-10 production; and JNK regulates IL-1 β , IL-10 and TNF- α production. It is of particular importance that the contribution of these MAPK families on the cytokine production is distinct among the agonists. For example, Erk 1/2 does not appear to contribute to CpG-ODN-induced IL-10 production (Fig. 4B). Neither p38, JNK nor Erk 1/2 is involved in LPS-induced IL-1 β production (Fig. 5).

TNF- α production in response to all agonists is inhibited with SB203580 (p38 inhibitor) and SP600125 (JNK inhibitor), but not with PD98059 within 3 hr. In most cases, the inhibitory effects persist for 24 hr with the particular exception that chitin-induced TNF- α production is not inhibited with SB203580 at 6 and 24 hr. It is possible that endogenous cytokines secondarily inter-regulate the cytokine production. For example, endogenous TNF- α appears to enhance IL-1 β and IL-10 production (6, 24). Cell activation induced by TNF- α

possibly involves p38, JNK, and Erk 1/2 (6, 7). On the other hand, IL-10 is a MØ deactivator that inhibits the synthesis of TNF- α , IL-1 β , IL-6, IL-8, IL-12, and GM-CSF in responding to LPS by human monocytes (8), mouse peritoneal MØ (11) and mouse splenic MØ (37). The literature indicates that IL-10 inhibits agonist-induced p38, Erk 1/2, and JNK activation (19, 21, 27, 33).

Previous studies (12, 14, 30, 43, 45) have shown that CLR-mediated phagocytosis induces inflammatory responses. For example, when MØ phagocytose zymosan particles through dectin-1/ β -glucan CLR, phagosomes include TLR2 and CLR-derived signals regulate TLR-derived signals (12, 30, 42). MØ production of IL-12p40 requires both activation of TLR2 and phagocytosis through dectin-1 simultaneously (12). Another example is that when immature dendritic cells are activated by LPS followed by phagocytosis of mannosylated lipoarabinomannan-coated beads through mannose receptors, LPS-induced IL-12 production is down-regulated whereas IL-10 production is up-regulated (14). The results suggest that CLR-derived signals play important regulatory roles in MØ phagocytosing microbes, although it remains to be elucidated whether these mechanisms are involved in chitin particle-induced MAPK activation and cytokine production.

In addition to specific CLR, cholesterol-rich microdomains (CRM) of MØ plasma membranes appear to be important to direct a choice of intracellular trafficking routes for invading intracellular pathogens (9). For example, virulent *Salmonella typhimurium* (5, 13), *Chlamydia trachomatis* (29), and *Brucella spp.* (28, 46) adhere to plasma membrane CRM and are internalized. This route of internalization results in immature phagosome formation, which allows bacterial replication in the MØ. In contrast, non-virulent bacteria are phagocytosed by MØ in a CRM-independent manner and induce microbicidal MØ development. Our studies also show that cholesterol depletion of RAW264.7 cells with methyl- β -cyclodextrin results in up-regulation of chitin-induced MAPK activation and the production of pro-inflammatory cytokines such as TNF- α (Manuscript in preparation). Furthermore, our preliminary studies showed that 1 – 4 μ m chitin particles induced five times higher IL-12 production by splenic MØ than 1 – 10 μ m chitin particles in equal masses (data not shown). Chitin particles at 1 – 4 μ m sizes contain twice as many particles as 1 – 10 μ m chitin particles in equal masses. Therefore, in addition to carbohydrate residues, the size and number of particles internalized appear to be the significant determinants.

In conclusion, our studies using RAW264.7 MØ-like cells have shown that when RAW264.7 cells phagocytose chitin particles, but not chitosan particles or latex beads, p38, Erk1/2, and JNK are activated. These activated MAPK families differentially regulate the production of TNF- α , IL-10, and IL-1 β . Future studies will seek to define the nature of cell surface receptor interactions and signaling, associated with the early stages of phagocytosis that modulate MØ-mediated host defense against intracellular bacterial infections.

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LEGENDS FOR FIGURES

FIG. 1. RAW264.7 cells produced TNF- α , IL-10, and IL-1 β when stimulated with chitin particles and control agonists. Cell suspensions at 5×10^5 cells/ml were added with 100 μ g/ml 1 – 10 μ m chitin particles, 100 μ g/ml soluble chitin oligosaccharide, 100 μ g/ml > 50 μ m chitin particles, 100 μ g/ml 1 – 10 μ m chitosan particles, 100 μ g/ml 1.1 μ m latex beads, 5 μ g/ml CpG-ODN, 5 μ g/ml GpC-ODN, 0.1 μ g/ml LPS, or saline. Cells were incubated at 37°C for 3, 6, and 24 hr. The levels of TNF- α (A), IL-10 (B), and IL-1 β (C) in culture supernatants were analyzed by ELISA. Mean \pm SD, $n=4$; *, $p < 0.05$; #, $p < 0.01$, compared to cells stimulated with saline at the same time point. The data shown are representative of two independent experiments.

FIG. 2. Chitin particles at 1 – 10 μ m sizes induced phosphorylation of p38, Erk1/2, and JNK in RAW264.7 cells. RAW264.7 cells at 1×10^6 cells/ml were stimulated with 1 – 10 μ m chitin particles (1,000 μ g/ml), soluble chitin oligosaccharide (1,000 μ g/ml), > 50 μ m chitin particles (1,000 μ g/ml), 1 – 10 μ m chitosan particles (1,000 μ g/ml), 1.1 μ m latex beads (1,000 μ g/ml), LPS (1 μ g/ml), CpG-ODN (5 μ g/ml), or GpC-ODN (5 μ g/ml) at 37 °C for 0, 10, 20, 30, and 40 min. One μ g protein for detection of p38 and Erk 1/2, and 4 μ g protein were separated for JNK detection on SDS-11% polyacrylamide gel and electroblotted to PVDF membrane. Total MAPK and dual phosphorylated MAPK were detected with specific antibodies. Pictures from one experiment representative of three are shown.

FIG. 3. The effects of MAPK pathway inhibitors on TNF- α production. Cells at 5×10^5 cells/ml were pre-treated with SB203580 (A), PD98059 (B), or SP600125 (C) at the indicated concentrations at 37 °C for 30 min. Cells were further stimulated with 100 μ g/ml 1 – 10 μ m chitin particles, 5 μ g/ml CpG-ODN, 0.1 μ g/ml LPS, or saline at 37°C for 3 hr. TNF- α levels in culture supernatants were determined by ELISA. Inhibitory effects on TNF- α production induced by chitin particles (D), LPS (E), or CpG-ODN (F) at 3, 6, and 24 hr after stimulation were also compared. Data (D – F) are indicated as % production compared to the production in culture without inhibitors at the same time point. Mean \pm SD, $n=3$; *, $p < 0.05$; #, $p < 0.01$, compared to cells stimulated with agonist in the absence of inhibitors at the same time point. The data shown are representative of two independent experiments.

FIG. 4. The effects of MAPK pathway inhibitors on IL-10 production. All cell preparations and treatments were identical to those in Fig. 3. IL-10 levels in the 24 hr-culture supernatants were measured by ELISA. Mean \pm SD, $n=3$; *, $p < 0.05$; #, $p < 0.01$, compared to cells stimulated with agonist in the absence of inhibitors at the same time point. The data shown are representative of two independent experiments.

FIG. 5. The effects of MAPK pathway inhibitors on IL-1 β production. All cell preparations and treatments were identical to those in Fig. 3. IL-1 β levels in the 24 hr-culture supernatants were measured by ELISA. Mean \pm SD, $n=3$; *, $p < 0.05$; #, $p < 0.01$, compared to cells stimulated with agonist in the absence of inhibitors at the same time point. The data shown are representative of two independent experiments.

FIG. 6. Schematic representation of chitin particle-induced MAPK activation regulating TNF- α (3 hr), IL-10 (24 hr), and IL-1 β (24 hr) production. Scheme of chitin particle-induced MAPK activation was proposed from

this study. CLRs represent mannose-type CLRs located on MØ plasma membrane. As comparison controls, signals that are induced by LPS and CpG-ODN are mediated by TLR4 and TLR9, respectively.

Fig. 1

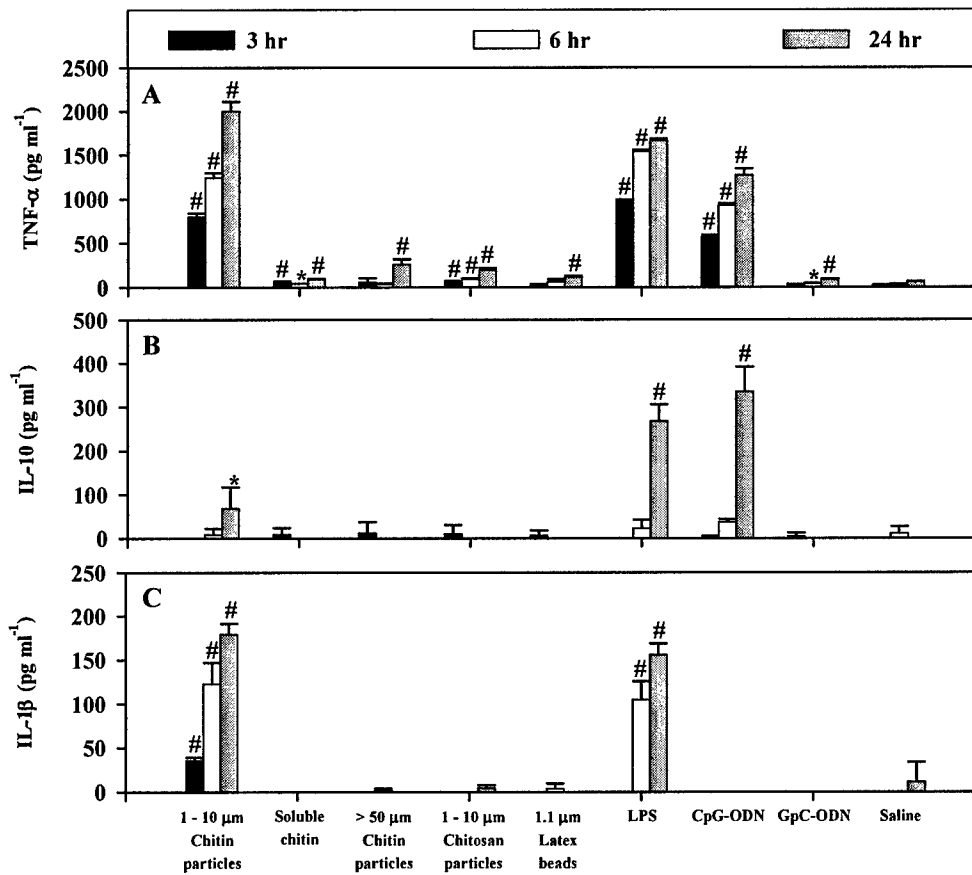


Fig. 2

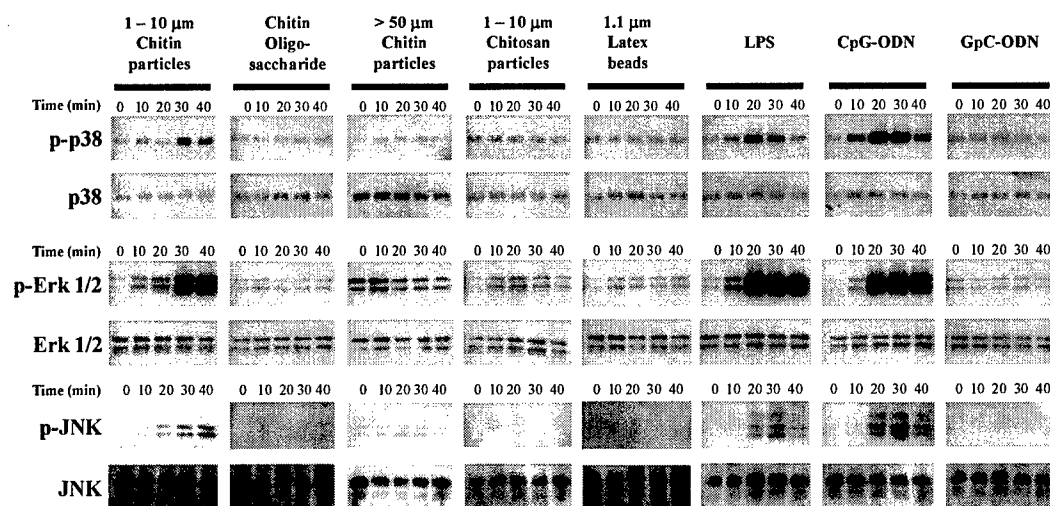


Fig. 3

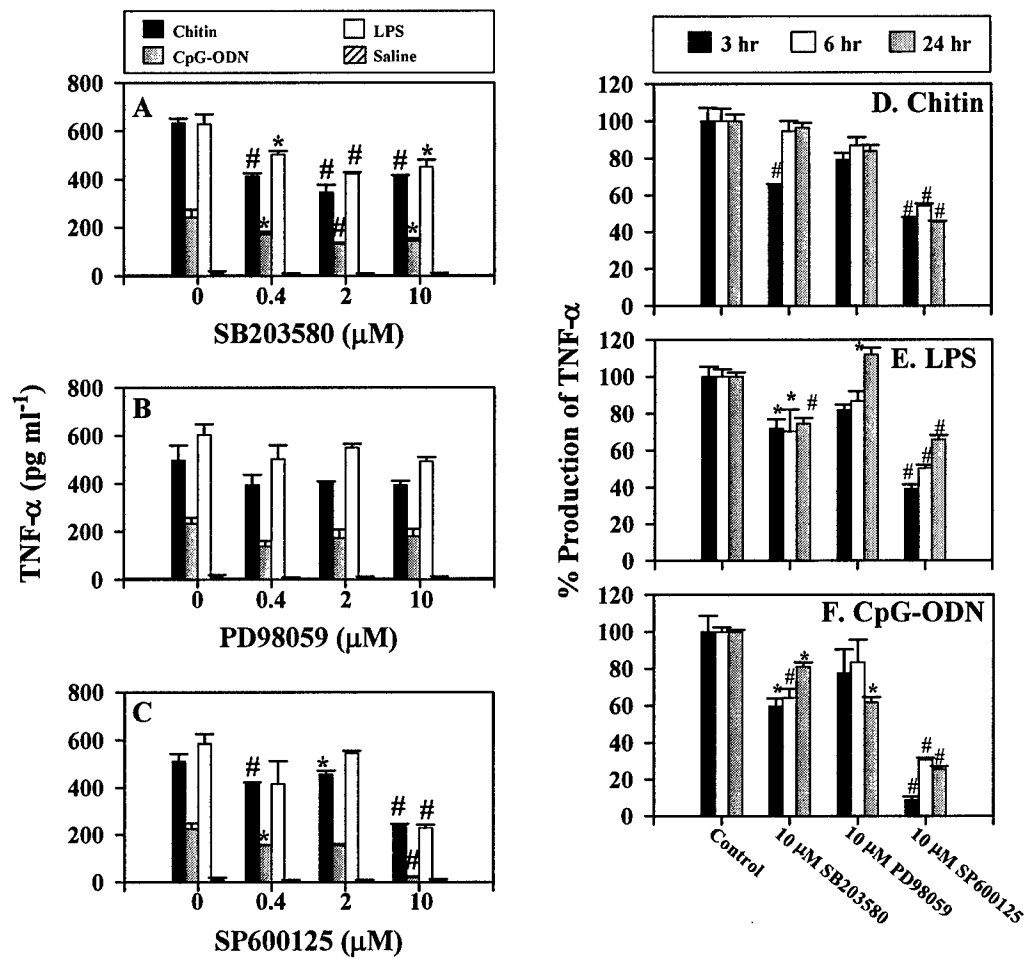


Fig. 4

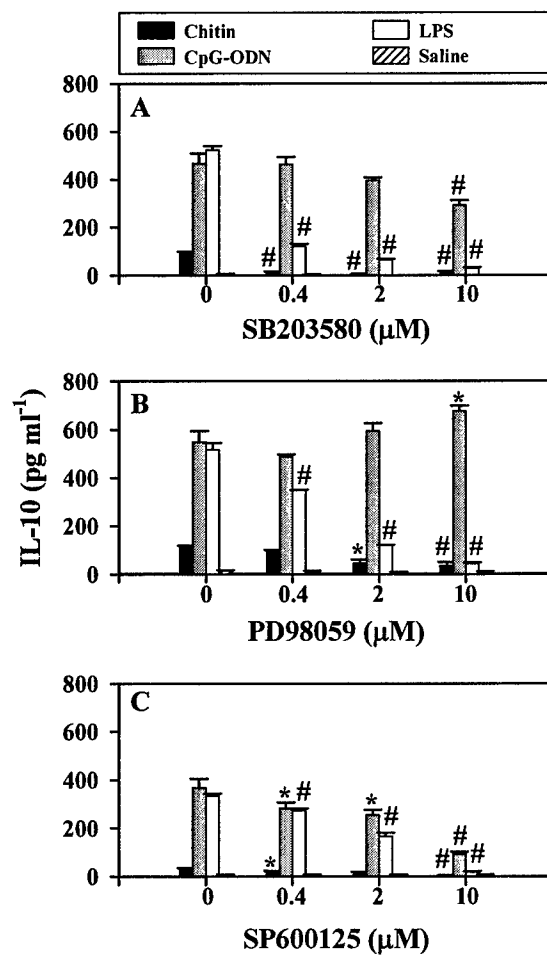


Fig. 5

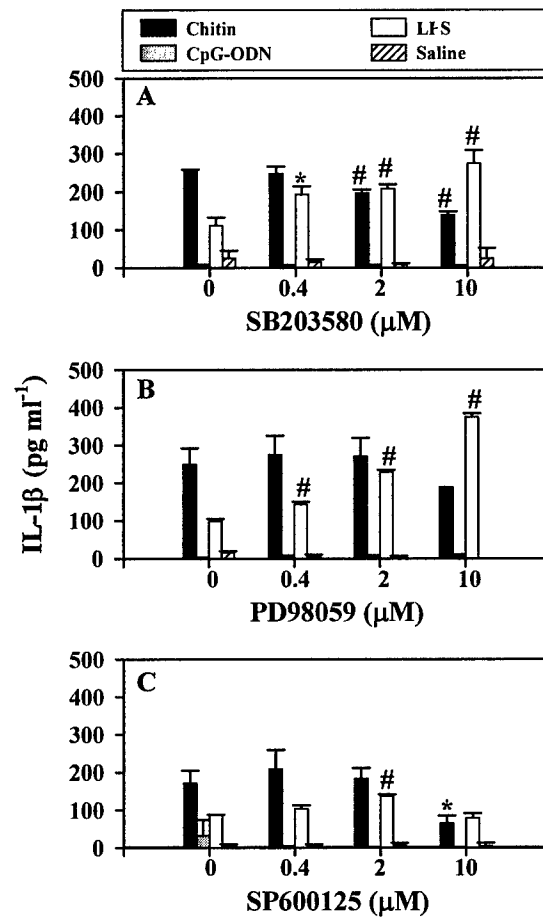
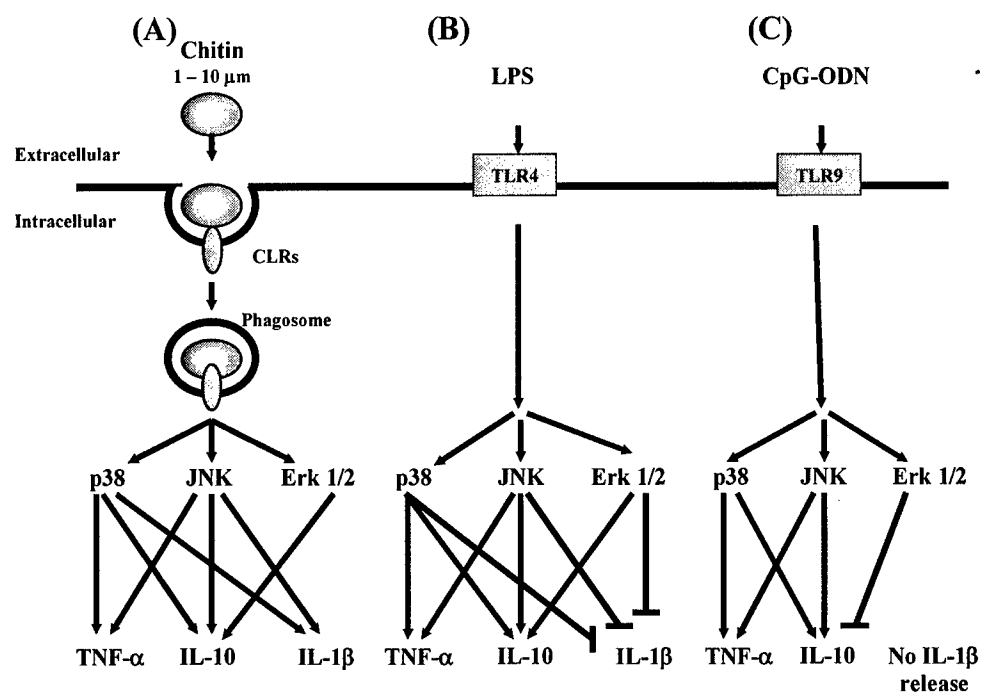


Fig. 6



Appendix IV

Marrow-derived splenic macrophages expressing Cox-2 may contribute to increased PGE₂ production in BCG-immunized mice

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Normal splenic macrophages (MØ) produce <1 ng PGE₂/10⁶ MØ/ml following ex-vivo stimulation. When mice are immunized with mycobacterial components (HK-BCG, 1 mg/dose, ip), splenic MØ released >10 ng PGE₂/ml at 7-21 days and facilitated a Th1-to-Th2 shift of immune responses. To understand the mechanism of splenic PGE₂-MØ formation, we characterized the expression of cellular Cox-2, a key enzyme for PGE₂ synthesis, in splenic MØ isolated from C57Bl/6 mice given HK-BCG ip. We found no Cox-2 expression by splenic MØ on Days 0 (normal), 3 and 4 after HK-BCG treatment. However, biphasic expression of Cox-2 was detected immunohistochemically and by Western blotting on Days 1 and 14. Furthermore, normal splenic MØ incubated with LPS expressed Cox-2 within 1 day. Neither 1-day splenic MØ prepared in vivo by HK-BCG, nor in vitro with LPS released significant amounts of PGE₂. A green fluorescent protein (GFP)-bone marrow chimera was established in C57Bl/6 recipients receiving bone marrow cells iv from GFP-donors. At 14-days after BCG immunization, GFP⁺ spleen cells were co-localized with Cox-2⁺ cells. We conclude that the presence of Cox-2 alone is not sufficient to induce PGE₂ production by the splenic MØ and that bone-marrow derived MØ may contribute to the increased PGE₂ production observed at 14 days after BCG immunization. (Supported by NIH HL 71711 & DOD PR023017)

Appendix V

Mycobacteria-induced osteoclastogenesis and PGE₂-releasing macrophage formation in the mouse spleen.

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Atherogenic lesions are often calcified possibly in association with bone resorption that would (is or may?) be accelerated by bacterial infections. The development of osteoclasts (OC) is regulated in bone marrow by osteoblasts and osteotropic factors such as PGE₂. However, the events leading to extramedullary osteoclastogenesis are largely unknown. In this study, we determined if BCG induces splenic hemopoiesis including OC and PGE₂-releasing macrophages (PGE₂-MØ).. Spleen cells were isolated from female Balb/c mice following immunization with heat-killed *Mycobacterium bovis* (BCG) at 1 mg/dose ip. The expression of the calcitonin receptor (CT-R), an 80 Kd OC antigen, was determined cytometrically and by Western blotting using anti-CT-R antibodies (Santa Cruz Biotech). PGE₂ levels released by splenic MØ *ex vivo* were measured by ELISA. We found that the levels of CT-R⁺ cells and PGE₂-MØ were increased at 5 days and reached to their peak along with increased hemopoiesis at one week. The levels declined slightly 3 weeks after BCG immunization. Our results indicate a temporal association of the two cell types in BCG-induced splenic hemopoiesis. It still remains to be investigated whether splenic osteoclastogenesis directly contributes to calcification of atherogenic lesions. (Supported by NIH HL 71711 & DOD PR023017)

Appendix VI

Program/Abstract #677.1

Mechanism of phagocytosed particle-induced IL-12 production in macrophage

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Chitin (N-acetyl-D-glucosamine polymer) can serve as a Th1 adjuvant. Previously, we found that macrophages (MØ) phagocytose 1 – 10 µm chitin particles through the mannose receptor, resulting in the production of IL-12, a Th1 cytokine, within 24 h. However, neither soluble chitin nor >50 µm chitin particles induce IL-12 production. To understand the signaling pathways involved in 1 - 10 µm chitin-induced IL-12 production, we examined the effects of SB203580 (p38 MAPK inhibitor), herbimycin A (PTK inhibitor) and cytochalasin D (actin polymerization inhibitor) on IL-12 production. Splenic MØ isolated from IL-10-KO mice were used to avoid the effect of IL-10, an inhibitor of IL-12 synthesis. LPS was used as a positive control since it induces IL-12 production via p38 MAPK activation. SB203580 at 0.2 µM and 0.3 µM inhibited chitin- and LPS-induced IL-12 production at 50%, respectively. Western blotting showed both chitin- and LPS-induced p38 MAPK phosphorylation within 20 min. Only chitin-induced IL-12 production was inhibited by 0.1 µM herbimycin A or 0.039 µM cytochalasin D. Our results indicate that IL-12 production in response to either phagocytosis of chitin or stimulation by LPS is dependent at least in part on p38 MAPK activation. However, the early stage of phagosome formation and PTK activation are preferentially linked to chitin-induced IL-12 production. (Supported by NIH HL71711 and DOD PR023017)

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Appendix VII

ORAL ADMINISTRATION OF N-ACETYL-D-GLUCOSAMINE POLYMER PARTICLES DOWN-REGULATES ALLERGIC RESPONSES

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BACKGROUND/PURPOSE: Children in military families are at high risk of asthma, and new strategies for reducing the incidence and health care costs are needed. Chitin is a naturally occurring N-acetyl-D-glucosamine polymer and new class of Th1 adjuvant that stimulates IL-12 production by macrophages *in vitro*. This study demonstrates that chitin down-regulates the allergic response in a murine model of allergic asthma.

METHODS: Ragweed-sensitized BALB/c mice were treated orally with saline or 1- 10 μ m chitin particles (8 mg/day for 3 days before and 13 days during ragweed allergen immunization, 7 mice per group). The mice were challenged with ragweed intratracheally on day 11. Three days after the challenge, serum IgE levels and lung eosinophil numbers were quantified. Th2 responses were further explored by measuring cytokine production by spleen cells isolated from the ragweed-immunized mice (controls) and cultured in the presence of ragweed and/or chitin for 3 days. **RESULTS:** The ragweed-sensitized mice treated with saline showed high levels of serum IgE and lung eosinophils, and splenocytes from these animals produced IL-4, IL-5, and IL-10 *in vitro*. Chitin treatment resulted in a significant reduction of these Th2 parameters ($p < 0.01$). **CONCLUSIONS:** Collectively, these results indicate that chitin, which induces innate immune responses, down-regulates Th2-facilitated IgE production and lung eosinophilia in the allergic mouse. Oral administration of chitin therefore represents a potentially effective treatment for IgE-mediated allergic diseases, including childhood asthma. Results of this study will support phase I trials on the effects of oral chitin on childhood asthma.

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